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13. ABSTRACT (Maximum 200 Words)

Lysophosphatidic acid (LPA) is a major mitogen in serum that regulates an array of cellular processes related to pathogenesis of cancer, especially ovarian, prostate and breast cancers. Interest in LPA has accelerated recently with the discovery that it is a ligand of a family of three G protein coupled cell surface receptors. Prostate cancer cells express these LPA receptors and it has been suggested that their expression correlates with more advanced prostate cancer. We found that androgen markedly upregulates expression of LPA(3) in LNCaP cells which are androgen-responsive prostate cancer cells, making them more similar to early stage carcinoma. In this grant period, we cloned a novel type of lipid kinase (MDGK) which phosphorylates monoacylglycerols and diacylglycerols to form LPA and PA, respectively. Both have been implicated in growth and survival of prostate cancer cells. Using a matched human tumor/normal tissue expression array, we found that MDGK expression was strikingly upregulated in prostate cancers compared to the normal prostate tissues from the same patient. In contrast, MDGK was similarly expressed in other types of cancers compared to their normal tissue counterparts, including kidney, breast, colon, and stomach cancers. MDGK is highly expressed in human prostate cancer cells, including androgen-responsive LNCaP cells, which are more similar to early stage carcinoma, and androgen-insensitive PTsu-Pr1 and PC-3 cells. Overexpression of MDGK in PC-3 cells results in secretion of LPA from these cells, transactivation of the epidermal growth factor (EGF) receptor, and subsequent activation of ERK1/2. Because of the well known role of the EGF receptor family in androgen-refractory metastatic prostate cancer, the pathophysiological significance of MDGK is to produce LPA which in turn can stimulate the release of mature EGF and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting this kinase, which is upstream of the EGF receptor, offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

14. SUBJECT TERMS
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INTRODUCTION

Originally known for its pedestrian role as an intermediate in intracellular lipid metabolism, LPA is now recognized as a potent lipid mediator that evokes growth-factor-like responses and regulates an array of cellular processes related to pathogenesis of cancer (1). These include stimulating proliferation by increasing cell cycle progression, enhancing cell survival, stimulating motility and inducing tumor cell invasion, and regulating neovascularization (1-5). Progress in understanding LPA actions has accelerated with the discovery that it is a ligand of several G protein coupled cell surface receptors (GPCRs), previously identified as members of the endothelial differentiation gene (EDG) family. To date, three LPA receptors have been identified, EDG-2/LPA_1, EDG-4/LPA_2, and EDG-7/LPA_3 (6-9). The LPA receptors are differentially expressed, coupled to a variety of G-proteins, and thus regulate diverse cellular responses (1,10,11). Intriguingly, expression of LPA receptors correlates with more advanced prostate cancer cell lines (12) and LPA_2 and LPA_3 are aberrantly expressed in ovarian cancer cells (13,14), indicating a potential role in the pathophysiology of cancer. Recently, a fourth LPA receptor was described (LPA_4/GPR23/P2Y9), which is distinct from the other LPA receptors (15). Moreover, LPA has a novel intracellular function as a high-affinity ligand for peroxisome proliferating activating receptor-γ, a transcription factor that regulates genes controlling energy metabolism (16).

In addition to actions through conventional GPCR signaling pathways, LPA receptors can indirectly regulate cell functions by transactivating the EGF tyrosine kinase receptor (17-19). This novel cross communication between different signaling systems is not only important for the growth promoting activity of LPA (17,19), it also may be a clue to its pathophysiological role in prostate cancer (18) and head and neck squamous cell carcinoma (20).

LPA is not only an active constituent of serum, it also accumulates to high concentrations in malignant effusions and has been proposed to be a marker and mediator of ovarian cancer progression (13,21,22). Although LPA is considered to be a key intermediate in de novo glycerolipid synthesis formed by acylation of glycerol 3-phosphate, abundant evidence now indicates that bioactive LPA is generated extracellularly. LPA can be produced from phosphatidic acid by phospholipase D (PLD) in activated platelets and ovarian and prostate cancer cells and subsequent deacylation by phospholipase A_1 or A_2 (23-25). The recent discovery that LPA is generated in the extracellular millieu from lysophosphatidylcholine by the enigmatic ecto-enzyme autotaxin, known to be involved in tumor invasion, neovascularization and metastasis (26,27), further supports the notion that LPA is an important regulator of tumor progression and metastasis (1,28).

Another potential pathway for synthesis of LPA is the phosphorylation of monoacylglycerols by a specific lipid kinase activity first described more than 30 years ago (29), which has received scant attention following the characterization of the partially purified bovine brain enzyme in 1989 (30,31).

BODY

Cloning a novel human lipid kinase

In the previous annual report, we described in detail the cloning of a lipid kinase that phosphorylates monoacylglycerols to form LPA and demonstrated that it is highly expressed in prostate cancers. We have now extensively characterized its enzymatic activity, localization, and signaling pathways that it modulates.

The new lipid kinase catalyzes the phosphorylation of monoacylglycerol to generate LPA

Although this new kinase was cloned based on its homology to SphKs, it displayed only very modest phosphorylating activity with sphingosine as substrate when compared to cells transfected with SphK1 or SphK2 (Fig. 1A). Moreover, unlike SphKs, neither D-*erythro*-dihydrosphingosine nor phytosphingosine were substrates for this kinase. In addition, there were no detectable changes in the levels of the sphingolipid metabolites, ceramide, sphingosine or S1P, in cells overexpressing this lipid kinase. We next examined *in vitro* kinase activity with an array of lipid substrates, including different ceramide species and glycerolipids, such as 1,2-dioleoyl-*sn*-glycerol (DAG), glycerol-3-phosphate, anandamide, phosphatidylinositol, phosphatidylglycerol, cardiolipin, and the monoacylglycerol, 1-oleoyl-2-*sn*-glycerol. Significant kinase activity was detected with monoacylglycerol and diacylglycerol, but not with any other lipid tested (Fig. 1B). Surprisingly, however, although the new kinase contains a DAGK catalytic domain, it did not have significant kinase activity with 1,2-dioleoyl-*sn*-glycerol as substrate when activity was measured in the presence of the detergent, octyl-β-glucopopyranoside, as usually used for DAGK activity measurements (32) (Fig. 1B), suggesting that the new lipid kinase is distinct from other known DAGKs. Moreover, when this lipid kinase was overexpressed in several types of cells, no changes in mass levels of diacylglycerol or phosphatidic acid could be detected (data not shown).

Previously, a monoacylglycerol kinase (MAGK) that was partially purified from bovine brain cytosol was reported to prefer substrates containing unsaturated fatty acid esters (30). Interestingly, the new lipid kinase has preference for substrates with a C18 fatty acid with one double bond, as monoacylglycerol with an oleoyl (18:1) substitution in the *sn1* position was phosphorylated to a greater extent than 1-palmitoyl-2-*sn*-glycerol (16:0), which was a better

substrate than 1-stearoyl-2-*sn*-glycerol (18:0) (Fig. 1B). Moreover, 1-*sn*-2-arachidonoyl-glycerol (2-AG), an endogenous cannabinoid receptor ligand (33,34), was also significantly phosphorylated (Fig. 1B). Like the crude bovine brain MAGK (30), this kinase required magnesium for maximal activity, whereas other divalent cations, including Ca^{2+} and Zn^{2+}, inhibited phosphorylation of 1-oleoyl-2-sn-glycerol. Similar to the brain MAGK, it also had higher activity in the presence of 0.03% deoxycholate, although activity was completely abolished by most other detergents, including Triton X-100, Triton X-114, CHAPS, and β-octylglucopyranoside (Fig. 1B and data not shown).

In the previous report, we identified the phosphorylated lipid produced by this lipid kinase *in vivo* as LPA. Because this new lipid kinase has a high degree of specificity for monoacylglycerol substrates and forms LPA *in vivo*, it is hereafter referred to as monoacylglycerol kinase or MAGK.

Subcellular localization of MAGK

Although it does not contain a mitochondrial localization signal, unexpectedly, confocal immunofluorescence microscopy revealed that MAGK was distributed in a punctate reticular pattern in NIH 3T3 cells (Fig. 2A), reminiscent of a mitochondrial localization. Indeed, there was no significant colocalization with cortical actin, stained with fluorescent phalloidin, the Golgi apparatus, stained with fluorescent WGA (data not shown), or with the ER marker calnexin as demonstrated by the absence of yellow color in the merged images (Fig. 2A). On the contrary, MAGK expression was clearly colocalized with MitoTracker stained mitochondria (Fig. 2A). Similar mitochondrial localization of MAGK was also observed in HEK 293 and PC-3 cells, indicating that the subcellular distribution was not cell-type specific.

To further substantiate the unusual localization of MAGK, protein expression and enzymatic activity were examined in subcellular fractions prepared by differential centrifugation. In agreement with the confocal results, by immunoblotting, a epitope tagged MAGK with the predicted MW of MAGK of 46.4 kDa was highly enriched in the P2 mitochondria fraction which was characterized by the presence of cytochrome c oxidase II (Fig. 2B). Much less MAGK was present in the P3 fraction containing intracellular membranes of the ER and Golgi. as determined with the specific ER marker PDI or in the P4 plasma membrane fraction, characterized by the presence of α_v-integrin (Fig. 2B). In concordance with the expression pattern of the protein, the highest MAGK specific activity was in the P2 fraction (Fig. 2C).

MAGK expression enhances cell growth

Growth promotion is one of the most prominent effects mediated by LPA (1). Indeed, transient or stable expression of MAGK enhanced proliferation of diverse cell types. The growth promoting effect of MAGK was observed even in the presence of sub-optimal serum concentrations (Fig. 3A). Analyses of the growth curves during the exponential phase revealed that MAGK expression decreased the doubling time from 37.7 h to 28 h. Addition of the MAGK substrate monoacylglycerol to cells cultured in serum-free medium significantly stimulated proliferation of MAGK expressing PC-3 cells but did not enable the vector transfectants to grow (Fig. 3B). In contrast, LPA increased proliferation of both MAGK and vector transfectants to the same extent (Fig. 3C).

MAGK promotes transactivation of EGFR

Many studies have led to the notion that LPA is important in the pathophysiology of prostate carcinoma functioning in an emerging paradigm of cross-talk between LPA receptors and the tyrosine kinase EGFR (1,17,18). Therefore, it was of importance to determine whether

overexpression of MAGK and increased LPA levels resulted in such receptor transactivation. In serum-starved cells, MAGK expression increased tyrosine phosphorylation of several proteins, notably a 170 kD band, which was similarly increased by serum in vector transfectants (Fig. 4A). Kinetic analysis of tyrosine phosphorylation in response to serum revealed that the 170 kD tyrosine phosphorylation was a rapid event in MAGK expressing cells, clearly evident within 5 min and remaining elevated for at least 60 min (Fig. 4D). Because LPA stimulates tyrosine phosphorylation of the EGFR in PC-3 cells (5,35), it was important to substantiate that the enhanced tyrosine phosphorylation of the 170 kD protein represented activation of the EGFR. Indeed, anti-phosphotyrosine immunoblotting of anti-EGFR immunoprecipitates revealed enhanced EGFR tyrosine phosphorylation in cells overexpressing MAGK, even in the absence of serum (Fig. 4C).

MAGK-induced ERK1/2 activation, motility, and IL-8 secretion requires EGFR

Previously, it has been suggested that EGFR activation is required for signal relay from LPA receptors to ERK1/2 in prostate cancer cells (18,35,36). MAGK expression markedly increased activation of ERK1/2, as determined with a phospho-specific antibody, which was further enhanced by serum (Fig. 4B) and EGF (Fig. 4F). To further confirm that activation of the EGFR was necessary for MAGK-stimulated ERK activation, we utilized the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478. AG1478 not only completely abolished EGFR-induced tyrosine phosphorylation (Fig. 4E), as expected, it also blocked MAGK-mediated ERK1/2 phosphorylation (Fig. 4F) and its mitogenic effect (data not shown). These results suggest that the tyrosine kinase activity of EGFR is required for MAGK-induced activation of the ERK cascade, culminating in DNA synthesis.

Transactivation of the EGFR has also been shown to be important for increased motility of cancer cells (20). In agreement, the enhanced migration of PC-3 cells towards EGF induced by expression of MAGK was blocked by the EGFR inhibitor AG1478 (Fig. 5A). In the Boyden chamber cell migration assay, differences in cell shape and size may affect passage through the pores in the membrane but do not affect the *in vitro* wound closure assay. MAGK expression enhanced closure of the wounded area, especially in the presence of EGF and MOG, which is phosphorylated by MAGK to form LPA (Fig. 5B,C). The wound closure enhanced by MAGK was also blocked by AG1478, supporting a role for EGFR transactivation in MAGK-induced migratory responses.

Expression of the multifunctional cytokine IL-8 correlates with angiogenesis, tumorigenicity, and metastasis of human prostate cancer cells implanted in nude mice (37). Moreover, LPA has been shown to stimulate IL-8 production in ovarian and colon cancer cells (38,39). Similarly, LPA markedly enhanced IL-8 secretion from PC-3 cells (Fig. 6). Addition of MOG stimulated IL-8 secretion in MAGK expressing cells by more than 2-fold but had no effect in vector transfected cells (Fig. 6). This increase in IL-8 was also decreased 60% by AG1478, and thus requires transactivation of the EGFR. In this regard, it has been shown that blockade of the EGFR in PC-3 cells not only inhibited tumor growth and invasion, it also downregulated expression of IL-8 within the tumors (40).

KEY RESEARCH ACCOMPLISHMENTS

- Cloning a monoacyglycerol kinase (previous report)
- Establishing the substrates for this kinase and cellular localization
- MAGK expression enhances cell growth

- MAGK expression enhances EGF-directed motility
- MAGK is involved in transactivation of EGFR
- MAGK is involved in ERK activation induced by EGF
- MAGK expression promotes IL-8 secretion

REPORTABLE OUTCOMES

1. Watterson, K., H. Sankala, S. Milstien, and **S. Spiegel.** 2003. Pleiotropic actions of sphingosine-1-phosphate. Prog. Lipid Res. 42:344-357.

2. **Spiegel, S.** and S. Milstien. 2003. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. Biochem. Soc. Trans. 31:1216-1219.

3. **Spiegel, S.** and S. Milstien. 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nature Rev. Mol. Cell Biol. 4:397-407.

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5. Bektas, M. and **S. Spiegel**. 2004. Glycosphingolipids and cell death. Glycoconj. J. 20:39-47.

Presentations

Lysophospholipids in Cancer. June 2003. Massey Cancer Center Research Retreat, MCV, Richmond, VA

Abstracts

A novel acylglycerol kinase that produces lysophosphatidic acid modulates crosstalk of growth signals in prostate cancer cells. Bektas, M., Payne, S.G., Liu, H., Milstien, S., Spiegel, S. Watt's Day Poster, Virginia Commonwealth University, 2003.

CONCLUSIONS

In this work, we have cloned the long searched for monoacylglycerol kinase (MAGK), a novel type of diacylglycerol kinase which phosphorylates monoacylglycerols, to form LPA. LPA has long been implicated as an autocrine and paracrine growth stimulatory factor for prostate cancer cells. The identification of this novel lipid kinase that regulates its production could provide new and useful targets for preventive or therapeutic measures. Because of the well-known role of the EGF receptor in androgen-refractory metastatic prostate cancer, the pathophysiological significance of our novel lipid kinase may be to produce LPA, which in turn can stimulate the release of mature EGF, and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting this kinase that is upstream of the EGF receptor offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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FIGURE LEGENDS

Figure 1. Lipid kinase activity of recombinant MAGK. (A) NIH 3T3 cells were transiently transfected with vector, hSphk1, hSphK2 or hMAGK. After 24 h, sphingosine phosphorylating activity was measured with 50 μM D-*erythro*-sphingosine substrate added as a BSA complex as described (41). (B) Lipid phosphorylating activity was determined in cell lysates from NIH 3T3 cells transiently transfected with vector (open bars) or hMAGK (filled bars). The following lipids were tested: C_6-cer; 1-oleoyl-2-*sn*-glycerol (18:1), MOG; 2-arachidonoyl-glycerol (20:4), 2-AG; 1-palmitoyl-2-*sn*-glycerol (16:0), MPG; 1-stearoyl-2-*sn*-glycerol (18:0), MSG; or diacylglycerol (1,2-dioleoyl-*sn*-glycerol), DAG. The data are expressed as pmol phosphorylated product formed/min/mg ± S.D. and are means of triplicate determinations. Where indicated, octyl-β-glucopyranoside was added to a final concentration of 1.5 %. Inset shows a TLC image of formed LPA band when MOG was used as substrate.

Figure 2. Subcellular localization of MAGK. (A) NIH 3T3 fibroblasts were transiently transfected with V5-tagged hMAGK and stained for mitochondria with MitoTracker Red. The endoplasmic reticulum was visualized with anti-calnexin antibody followed by FITC-conjugated anti-rabbit as the secondary antibody. hMAGK was stained with monoclonal anti-V5 antibody followed by secondary FITC-conjugated or Texas Red-condugated anti-mouse antibody. Cells were visualized by dual wavelength confocal microscopy. Superimposed merged pictures are shown in the lower panels, yellow color indicates colocalization. (B, C) **Activity and expression of MAGK in subcellular fractions.** Lysates from HEK 293 cells transfected with vector or V5-MAGK were fractionated into P1 (nuclei and unbroken cells), P2 (mitochondria), P3 (ER, and Golgi), P4 (plasma membrane) and cytosol. Proteins (25 μg) were resolved by SDS-PAGE and immunoblotted with anti-V5, anti-cytochrome c oxidase, anti-phosphodisulfide

isomerase (PDI) or anti-a_v-integrin as specific organelle markers. (C) MAGK activity was also determined in each fraction with MOG as substrate.

Figure 3. MAGK stimulates proliferation. PC-3 prostate cancer cells stably transfected with vector or hMAGK were cultured in serum-free medium supplemented with 0.5% serum (A), 10 μM MOG (B) or 10 μM LPA (C) and cell numbers determined. Asterisks denote significant differences ($p < 0.01$, Student t test). Similar results were obtained in two additional experiments.

Figure 4. Enforced expression of MAGK enhances EGFR tyrosine phosphorylation and stimulates ERK1/2. Serum-starved PC-3 cells stably transfected with vector or hMAGK, were stimulated without or with 10 % FBS for 10 min (A, B, C) or the indicated times (D), lysed and immunoblotted with anti-phosphotyrosine (A, D) or phospho-specific anti-ERK1/2 antibodies (B). Blots were then stripped and re-probed with ERK 2 antibody (B) or anti-tubulin (D) to demonstrate equal loading. (C) Lysates from cells treated as in (A) were immunoprecipitated with anti-EGFR antibody and the immunoprecipitates analyzed by western blotting using anti-phosphotyrosine or anti-EGFR antibody. (E, F) MAGK expression induces EGFR transactivation. Serum-starved PC-3 cells stably transfected with vector or hMAGK were preincubated for 20 min in the absence or presence of 250 nM tyrphostin (AG1478), then treated with EGF for 10 min. Cell lysate proteins were analyzed by immunoblotting with (E) anti-phosphotyrosine or with (F) phospho-specific ERK1/2. Blots were stripped and re-probed with ERK 2 antibody to demonstrate equal loading.

Figure 5. MAGK-stimulated cell migration towards EGF and wound closure require EGFR. (A) PC3 cells transfected with vector (open bars) or hMAGK (filled bars) were pretreated without or with AG1478 (200 nM) for 20 min and then allowed to migrate for 4 h

towards EGF (10 ng/ml). The data are means ± S.D. of three individual determinations. (B,C) Vector or MAGK transfected PC3 cell monolayers were scraped and treated with vehicle, MOG (10 μM), LPA (10 μM), or EGF (10 ng/ml) in the absence or presence of AG1478 (200 nM) and migration of cells into the wound was determined by processing digital photographs with Image Pro after 24 h. (C) Typical wound healing assay of vector and MAGK transfected PC3 cells before and after 24 h treatment with MOG.

Figure 6. MAGK induces IL-8 secretion. PC3 cells transfected with vector (open bars) or MAGK (filled bars) were serum-starved for 12 h and then treated in serum-free DMEM with MOG (10 μM) or LPA (10 μM) for 16 h. IL-8 secretion was determined by ELISA.

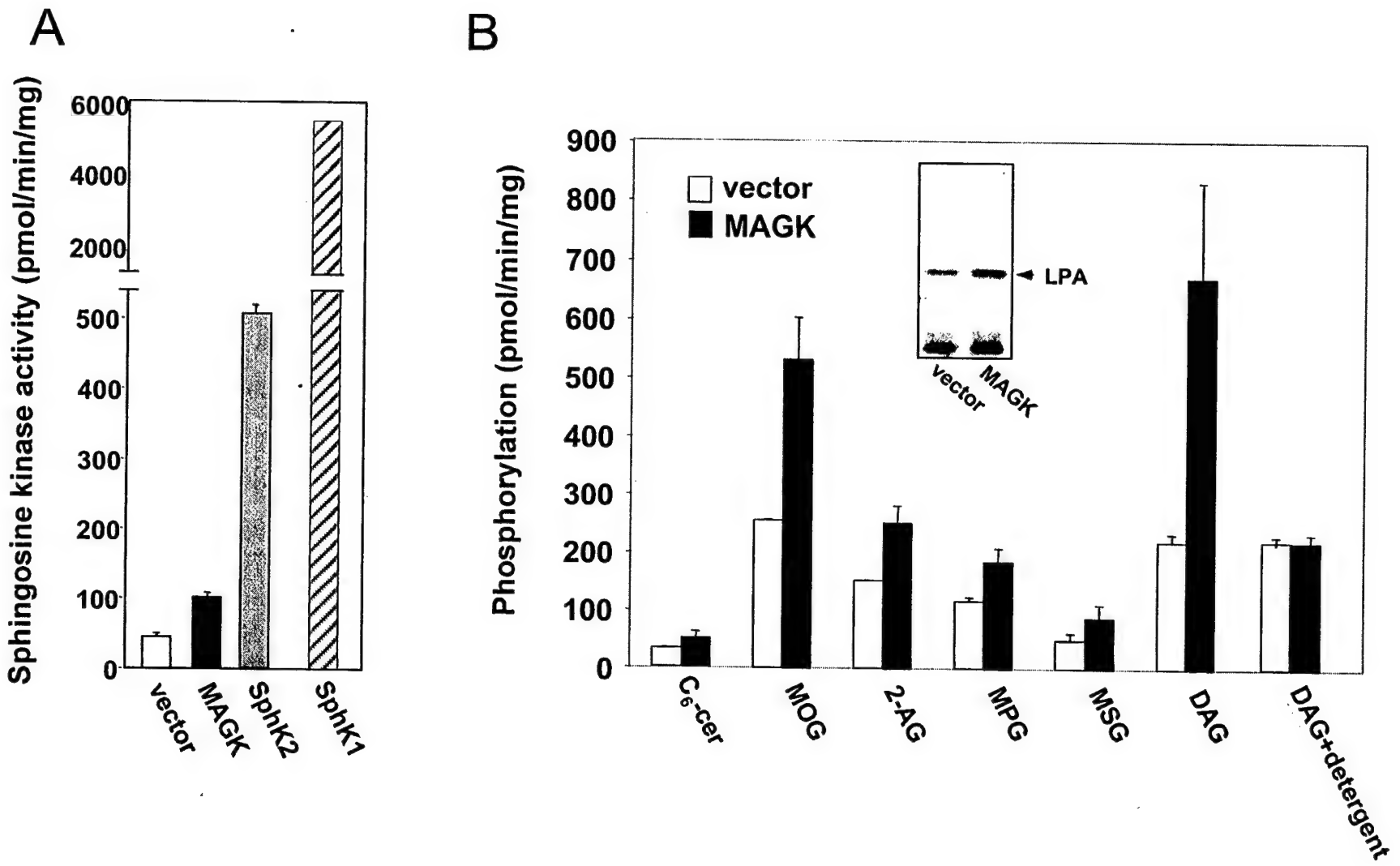
A
Sphingosine kinase activity (pmol/min/mg)
6000
4000
2000
500
400
300
200
100
0
vector
MAGK
SphK2
SphK1
B
Phosphorylation (pmol/min/mg)
900
800
700
600
500
400
300
200
100
0
vector
MAGK
LPA
vector
MAGK
C6-cer
MOG
2-AG
MPG
MSG
DAG
DAG+detergent

Figure 1

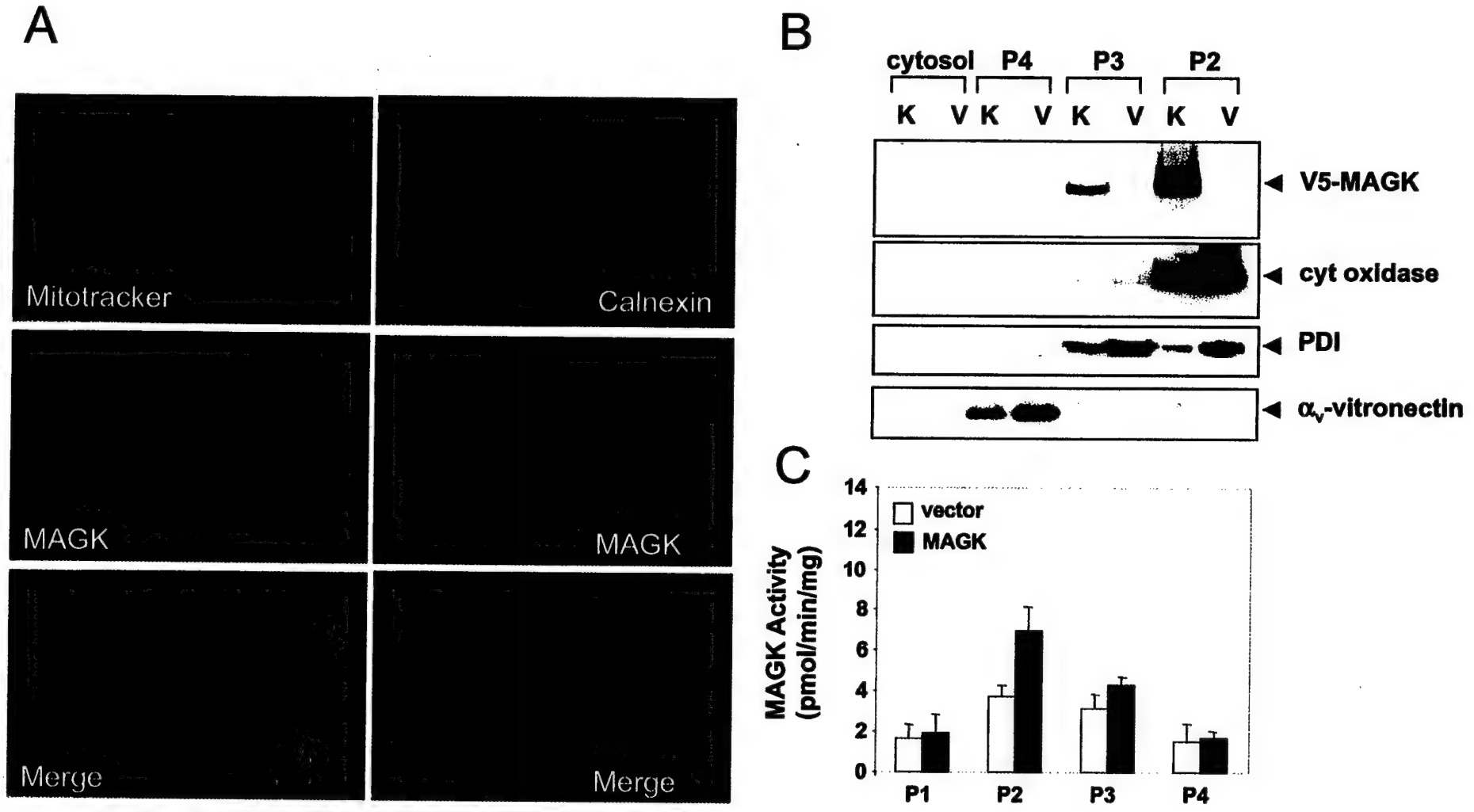
A
Mitotracker
Calnexin
MAGK
MAGK
Merge
Merge
B
cytosol
P4
P3
P2
K V K V K V K V
V5-MAGK
cyt oxidase
PDI
αv-vitronectin
C
MAGK Activity (pmol/min/mg)
vector
MAGK
0
2
4
6
8
10
12
14
P1
P2
P3
P4

Figure 2

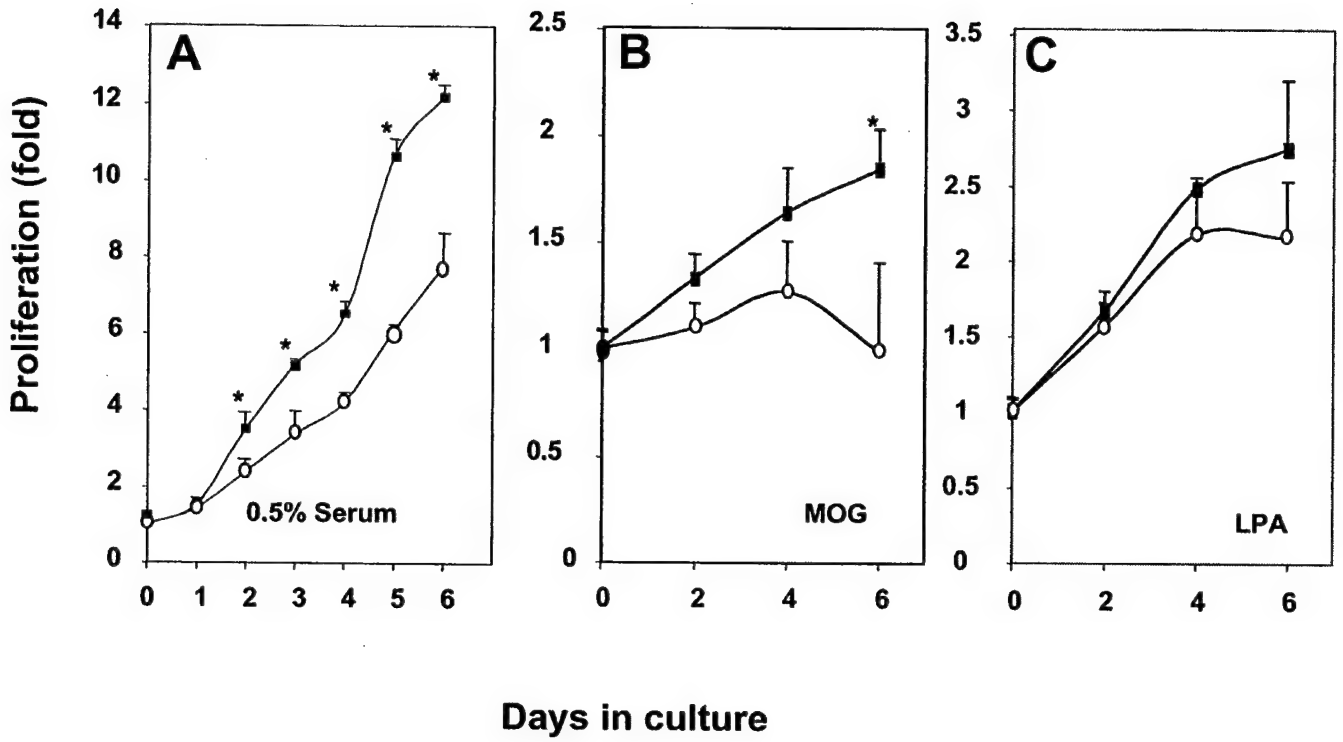

Figure 3

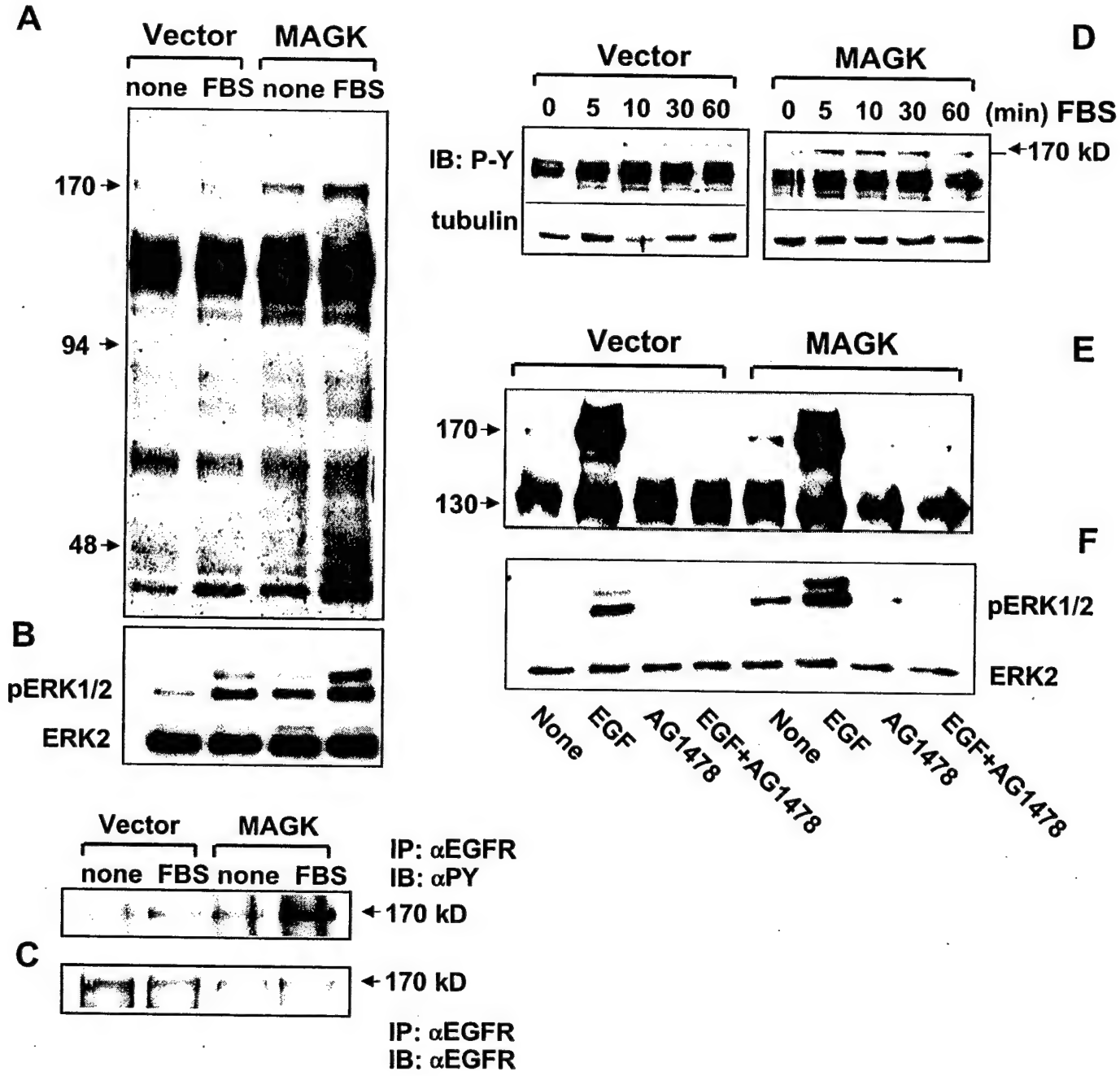
A
Vector
MAGK
none FBS none FBS
170
94
48
B
pERK1/2
ERK2
C
Vector
MAGK
none FBS none FBS
IP: αEGFR
IB: αPY
170 kD
170 kD
IP: αEGFR
IB: αEGFR
D
Vector
MAGK
0 5 10 30 60
0 5 10 30 60 (min) FBS
IB: P-Y
tubulin
170 kD
E
Vector
MAGK
170
130
F
pERK1/2
ERK2
None
EGF
AG1478
EGF+AG1478
None
EGF
AG1478
EGF+AG1478

Figure 4

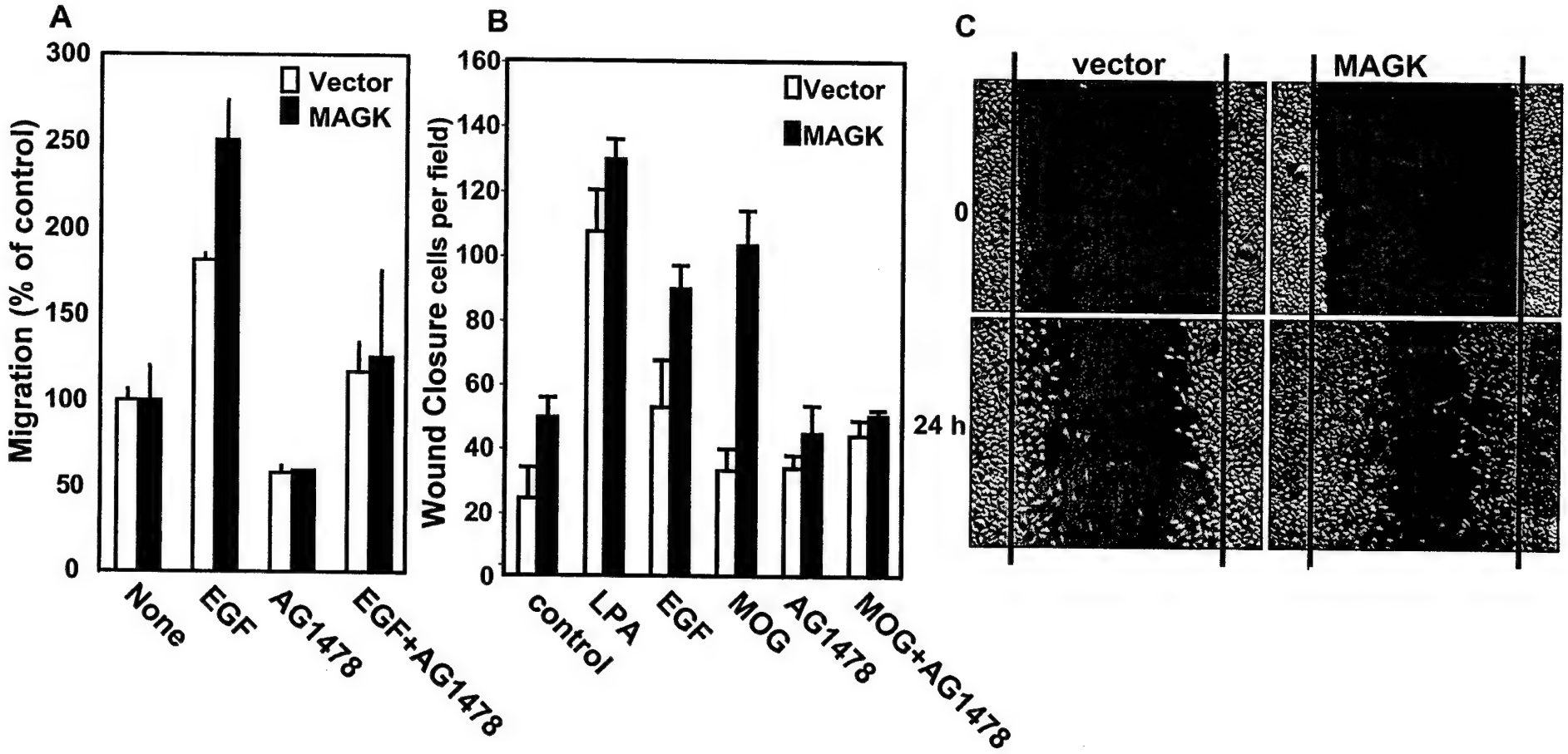
A
Migration (% of control)
300
250
200
150
100
50
0
Vector
MAGK
None
EGF
AG1478
EGF+AG1478
B
Wound Closure cells per field)
160
140
120
100
80
60
40
20
0
Vector
MAGK
control
LPA
EGF
MOG
AG1478
MOG+AG1478
C
vector
MAGK
0
24 h

Figure 5

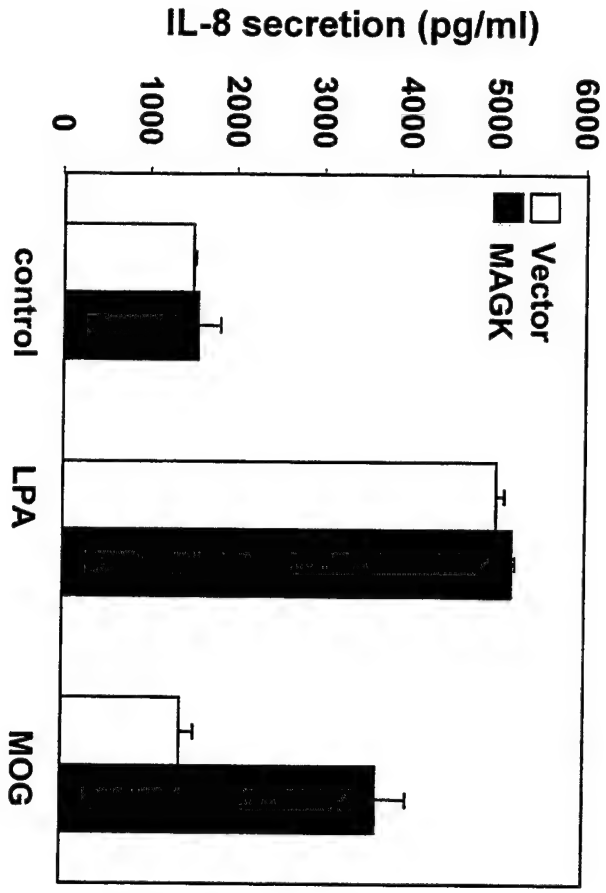
IL-8 secretion (pg/ml)
0
1000
2000
3000
4000
5000
6000
Vector
MAGK
control
LPA
MOG

Figure 6

APPENDIX 1

Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways

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Abstract

S1P (sphingosine 1-phosphate) is the ligand for a family of specific G-protein-coupled receptors that regulate a wide variety of important cellular functions, including vascular maturation, angiogenesis, cell growth, survival, cytoskeletal rearrangements and cell motility. However, S1P also may have intracellular functions. In this review, we discuss two examples that clearly indicate that intracellularly generated and exogenous S1P can regulate biological processes by divergent pathways.

Introduction

S1P (sphingosine 1-phosphate), a sphingolipid metabolite found in organisms as diverse as plants, yeast, nematode worms, flies and mammals, is a potent regulator of a wide spectrum of important biological processes [1]. S1P is produced by phosphorylation of sphingosine catalysed by SphK (sphingosine kinase), a highly conserved enzyme that is activated by many agonists and stimuli [1,2]. S1P actions are mediated by binding to a family of five specific G-protein-coupled receptors, $S1P_1$–$S1P_5$, which are differentially expressed and coupled to a variety of G-proteins [1,3–5]. There is no doubt that the most important functions of S1P are mediated through its receptors, and these include regulation of angiogenesis, vascular maturation, cardiac development, neuronal survival and immunity [1,3,4]. The importance of these receptors has been most clearly shown by mutations and disruptions of S1P receptor genes. For example, mutations in $S1P_2$ lead to abnormal split heart development in the zebrafish [6]. Moreover, deletion of $S1P_1$ in mice revealed that it is essential for vascular maturation and migration of smooth muscle cells and pericytes around newly formed endothelium [7]. An exciting recent development with important clinical implications for S1P receptor signalling was the finding that the immunosuppressive drug FTY720, a sphingosine analogue, is phosphorylated by SphK to an active phosphorylated form which then, acting via S1P receptors, induces lymphopenia [8,9]. These studies implied a role for S1P receptors in lymphocyte homing and immunoregulation.

Other studies, however, suggested that S1P also has second messenger functions important for regulation of calcium homoeostasis [10,11], cell growth [12–14] and suppression of apoptosis [15–17]. Dissection of the intra- and extracellular actions of S1P is difficult, as intracellular targets have not been definitively identified and it has been shown that S1P, by binding to its receptors, can stimulate SphK to increase its own intracellular levels [18]. On the other hand, growth factors, such as PDGF (platelet-derived growth factor), can bind to their receptors to activate and recruit SphK to the leading edge of the cell [19], where it produces S1P to spatially and temporally stimulate cell-surface $S1P_1$ in an autocrine or paracrine manner [20] leading to activation of downstream signals crucial for cell movement [19,20]. It has been proposed that the PDGF receptor is in fact tethered to $S1P_1$, providing a platform for integrative signalling by these two types of receptor [21]. Another complicating factor in discriminating between intra- and extracellular actions of S1P is the fact that it can be specifically transported into cells by the cystic fibrosis transmembrane regulator (CFTR) [22], which could function to terminate extracellular signals as well as to initiate intracellular signals. In this review, we will describe two examples that show that exogenous and intracellularly generated S1P can affect cellular processes by distinct pathways.

SphK1 and generation of intracellular S1P, but not exogenous S1P, potentiate TNF-α (tumour necrosis factor α)-stimulated BH_4 (tetrahydrobiopterin) biosynthesis in C6 glioma cells

In astroglial cells, the biosynthesis of BH_4, the coenzyme required for NO synthesis and hydroxylation of tyrosine and tryptophan, is stimulated by various proinflammatory cytokines, including TNF-α. These induce expression of GTP cyclohydrolase, the rate-limiting enzyme in the *de novo* pathway for BH_4 biosynthesis. We observed that TNF-α stimulates iNOS (inducible nitric oxide synthase), which

Key words: cell growth and survival, G-protein-coupled receptor (GPCR), sphingosine kinase, sphingosine 1-phosphate (S1P), sphingosine 1-phosphate receptor, tetrahydrobiopterin.

Abbreviations used: BH_4, tetrahydrobiopterin; ERK, extracellular-signal-regulated kinase; S1P, sphingosine 1-phosphate; SphK, sphingosine kinase; PDGF, platelet-derived growth factor; TNF-α, tumour necrosis factor α; iNOS, inducible nitric oxide synthase; dihydro-S1P, sphinganine 1-phosphate.

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requires BH_4 for its activity, and GTP cyclohydrolase expression by divergent pathways [23]. Whereas TNF-α stimulated iNOS expression via a ceramide-dependent pathway, TNF-α regulated GTP cyclohydrolase expression independently of ceramide generation, and the sphingolipid metabolite S1P, but not ceramide, potentiated GTP cyclohydrolase mRNA expression induced by TNF-α. Conversely, TNF-α-induced SphK activation and generation of S1P did not modulate expression of iNOS. Thus TNF-α induces the co-ordinate expression of iNOS and GTP cyclohydrolase via ceramide- and S1P-dependent pathways, respectively [23]. Differential regulation of iNOS expression and levels of its cofactor BH_4 may be physiolologically relevant, as BH_4 has other important functions, especially in hydroxylation of the aromatic amino acids. Thus cytokine-dependent induction of iNOS and BH_4 synthesis should be co-ordinately regulated only in cell types where NO production is important.

As TNF-α stimulates GTP cyclohydrolase expression via a ceramide-independent pathway [23] and also increases S1P levels [24–26], it was of interest to investigate the role of SphK and S1P in the regulation of GTP cyclohydrolase and BH_4 biosynthesis. We found that stimulation of SphK and generation of intracellular S1P by TNF-α was involved in the regulation of GTP cyclohydrolase expression and activity, as the SphK inhibitor, *N,N*-dimethylsphingosine, completely blocked the potentiation effect of SphK on TNF-α-induced GTP cyclohydrolase and BH_4 synthesis [27]. Remarkably, exogenous S1P or dihydro-S1P (sphinganine 1-phosphate), which both bind to and activate all of the S1P receptors, did not mimic the effect of overexpression of SphK and increased intracellular S1P on GTP cyclohydrolase expression and activity or on BH_4 biosynthesis [27]. This was not due to a failure to activate S1P receptors on C6 cells, as binding of S1P and dihydro-S1P to $S1P_1$ present on C6 cells markedly activated ERK (extracellular-signal-regulated kinase). It was previously shown that activation of ERK by S1P leads to induction of expression of Egr-1, one of the immediate early gene products required for expression of this essential transcription factor for fibroblast growth factor-2, an autocrine factor that stimulates proliferation of astroglial cells [28,29]. Interestingly, overexpression of SphK1 not only potentiated TNF-α-induced BH_4 biosynthesis, but also, similar to its effect on fibroblasts [14,30], enhanced proliferation of C6 cells. However, whereas this proliferative effect could be blocked by PD90859, an inhibitor of the ERK pathway, similar treatment had no effect on TNF-α-induced BH_4 biosynthesis. Thus, although some effects of S1P, such as ERK activation, are clearly mediated through interactions with S1P receptors, BH_4 biosynthesis is only regulated by intracellularly generated S1P, not by exogenous S1P, and GTP cyclohydrolase may be an intracellular target of S1P.

One of the important functions of astroglial cells is to support neuronal cells by secreting a variety of neurotrophic factors, such as fibroblast growth factor-2 and other peptide growth factors, and neurotransmitters [31]. It should be noted that S1P can act in an autocrine or paracrine manner through different members of the S1P receptor family present on both astroglial and neuronal cells. S1P may therefore be another factor provided by glial cells *in vivo* to promote neuronal cell survival and morphology rearrangements and remodelling of the actin cytoskeleton during various stages of development. Alternatively, it could have pathophysiological effects at sites of brain lesions and alter the blood–brain barrier [32].

SphK promotes growth and survival independent of S1P receptors

Expression of SphK1 elevated intracellular levels of S1P, expedited the G_1/S transition, protected against apoptosis [14] and enhanced tumour formation in mice [30,33]. Heterotrimeric G-proteins couple cell-surface receptors to signals that regulate proliferation and survival, and asynchronous activation of Gα subunits can lead to oncogenic transformation [34]. Moreover, in some cases it is the Gβγ dimers that mediate proliferation via ERK1/2 activation and promote cell survival by activation of phosphoinositide 3-kinase [35]. The many studies implicating G-protein-coupled S1P receptors in the biological activities of S1P have overshadowed its intracellular roles, mainly due to the difficulty of dissociating signals that originate at the cell surface from those potentially originating inside cells. Pertussis toxin, which ADP-ribosylates and inactivates G_i proteins, has frequently been used to implicate a $G\alpha_i$-mediated pathway in proliferative and survival effects induced by exogenous S1P [13,36,37]. However, while pertussis toxin did not affect the proliferation and cytoprotective effects induced by SphK1 overexpression, these were completely blocked by a SphK inhibitor [14]. In agreement, inhibiting α_i and α_q, but not $\alpha_{12/13}$, drastically reduced proliferation and ERK1/2 activation induced by exogenous S1P. In sharp contrast, blocking signalling of the various Gα subunits and Gβγ dimers, the G-proteins that S1P receptors couple to and signal through, did not influence growth and survival promoted by SphK1 and intracellularly generated S1P [38]. Thus although the mitogenic effect of exogenous S1P appears to be mediated by binding to cell-surface S1P receptors, S1P formed by overexpression of SphK1 promoted growth and survival independently of these receptors. In further support of this conclusion, expression of SphK1 markedly stimulated growth of pertussis toxin-treated $S1P_2$/$S1P_3$ double-knockout mouse embryonic fibroblasts, which then have no functional S1P receptors [38]. In contrast with the strong mitogenic effect of SphK1 in pertussis toxin-treated $S1P_2$/$S1P_3$ double-knockout embryonic fibroblasts, no significant responses were observed with exogenous S1P [38]. These results suggest that S1P receptors are dispensable for the mitogenic effect of SphK1 but contribute to that of exogenous S1P. Moreover, pertussis toxin slightly reduced the protective effects of serum and high concentrations of S1P. In contrast, pertussis toxin did not decrease the strong cytoprotective effect of SphK1 overexpression in either wild-type or S1P receptor-null embryonic fibroblasts [38]. In summary, even in the absence of all S1P receptor signalling, SphK1 still markedly induced growth and survival. These data indicate that exogenous and intracellularly generated

S1P affect cell growth and survival by divergent pathways. Moreover, although intracellularly generated S1P can signal inside-out to regulate cytoskeletal rearrangements and cell movement, this is not the case for the regulation of cell growth and suppression of apoptosis, which is independent of S1P receptors.

Several other lines of evidence further support the concept of intracellular actions of S1P. First, dihydro-S1P, which is identical to S1P and only lacks the 4,5-*trans* double bond, binds to all of the S1P receptors, yet does not mimic the effects of S1P on cell survival [13,16,19,24]. Secondly, elevation of intracellular S1P by microinjection mobilizes calcium [11] and enhances proliferation and survival [13,16,39]. Thirdly, SphK1 and conversion of sphingosine into S1P mediates vascular endothelial growth factor-induced activation of Ras leading to activation of the ERK pathway and cell division by inhibiting the GTPase-activating protein, RasGAP, without the participation of S1P receptors [40]. Fourthly, levels of phosphorylated long-chain sphingoid bases regulate yeast environmental stress responses and survival although they do not have any S1P receptors [41–43], in a manner reminiscent of the function of S1P in eukaryotic cells. Accumulated sphingoid bases in yeast induce G_0/G_1 arrest, and the yeast SphKs, Lcb4 and Lcb5, remove the sphingoid block, allowing progression to S phase [43]. Similarly, PDGF-induced activation of CDK2, a cyclin-dependent kinase that promotes cell-cycle progression, was dependent on SphK [44]. This might be due to nuclear formation of S1P, as the time course for CDK2 activation is similar to that of the increase in nucleoplasmic SphK activity and translocation to the nuclear envelope induced by PDGF [45]. Finally, S1P regulates guard cell aperture in plants, which do not have S1P receptors, by direct effects on heterotrimeric Gα proteins. Recent studies with the tobacco plant *Arabidopsis thaliana* suggest that S1P links the drought hormone, abscisic acid, to regulation of stomatal aperture and guard cell ion channels [46]. Interestingly, S1P regulated stomatal apertures and guard cell inward K^+ channels and slow anion channels only in wild-type *Arabidopsis* cells, but not in cells with a knockout of GPA1, their only heterotrimeric G-protein α subunit gene [46]. These results suggest that S1P may be able to activate G-proteins directly, independently of cell-surface receptors. Whether S1P can also activate heterotrimeric G-proteins in eukaryotic cells in a similar fashion remains to be determined.

In conclusion, the concept of intracellular actions of S1P independent of S1P receptors and 'inside-out signalling' is not only important for understanding S1P functions, but may have therapeutic implications for development of SphK inhibitors for treatment of cancer [30] and also for prevention of radiation-induced premature ovarian failure and infertility [16,39].

We thank the members of the Spiegel laboratory and particularly Dr Ana Olivera and Dr Lewis Vann for contributions to the studies that were discussed in this review. This work was supported by research grants to S.S. from the National Institutes of Health (GM43880, CA61774, AI50094) and the Department of the Army (DAMD17-02-1-0060).

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Review

Pleiotropic actions of sphingosine-1-phosphate

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that regulates diverse cellular responses including, growth, survival, cytoskeleton rearrangements and movement. S1P plays an important role during development, particularly in vascular maturation and has been implicated in pathophysiology of cancer, wound healing, and atherosclerosis. This review summarizes the evidence showing that signaling induced by S1P is complex and involves both intracellular and extracellular actions. The intracellular effects of S1P remain speculative awaiting the identification of specific targets whereas the extracellular effects of S1P are clearly mediated through the activation of five specific G protein coupled receptors, called $S1P_{1-5}$. Recent studies demonstrate that intracellular generated S1P can act in a paracrine or autocrine manner to activate its cell surface receptors.

Contents

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membrane ruffling and cell migration [68]. These observations may be physiologically relevant as $S1P_2$ is expressed in cells in which S1P inhibits cell migration, such as melanoma and vascular smooth muscle cells [68].

7. $S1P_4$ and $S1P_5$

$S1P_4$ and $S1P_5$ are the most recently identified and therefore the least well characterized S1PRs. $S1P_4$ has a highly restricted expression pattern, being expressed primarily in lymphoid and hematopoeitic tissues, as well as in the lung [38,56]. $S1P_5$ is expressed in a variety of tissue types but is highly expressed in the white matter of the brain and in the spleen [38,56,69,70]. $S1P_4$ mediates S1P-induced PLC activation, intracellular Ca^{2+} mobilization, and MAPK activation, in a PTX-sensitive manner [38]. $S1P_5$ couples to $G_{i/0}$ and G_{12} but not to G_s or $G_{q/11}$ [69,71]. In CHO cells transfected with $S1P_5$, S1P-inhibited forskolin-induced cAMP accumulation was PTX-sensitive while activation of JNK and inhibition of serum-induced activation of ERK1/2 was PTX-insensitive [71]. The inhibitory effect of S1P on ERK1/2 activity was abolished by treatment with orthovanadate, suggesting the involvement of a tyrosine phosphatase [71].

8. S1PR signaling in angiogenesis

One of the most important biological roles of S1PRs is in angiogenesis, the process of new blood vessels formation from pre-existing ones. This process is an integral component of many physiological events, such as embryonic development, wound healing, and the menstrual cycle, each of which are defined by a requirement for new vessel formation to simultaneously supply oxygen and nutrients [72]. Angiogenesis is also critically important in a number of pathological conditions associated with blood vessel formation, including solid and hematologic tumor progression, chronic inflammation in rheumatoid arthritis and Crohn's disease, endometriosis, and diabetic retinopathy [72]. The process of angiogenesis involves a number of steps; 1) initiation; 2) endothelial cell migration and proliferation; 3) differentiation; and 4) maturation of the neovasculature. Recent studies have suggested that these steps are regulated by S1P-dependent activation of $S1P_1$ [47,50,55,57,73,74].

9. Initiation of angiogenesis

Vascular endothelial growth factor (VEGF), an important mediator of angiogenic initiation, is known to act on VEGF receptors (VEGFRs) to induce vasodilatation via NO production and increased endothelial cell permeability, allowing plasma proteins to enter the tissue and form a fibrin-rich provisional network. To date, there are three known VEGF tyrosine kinase receptors; VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4). VEGFR-1 and VEGFR-2 are expressed mainly in the vascular endothelium whereas VEGFR-3 is mostly restricted to the lymphatic endothelium [72]. Recent studies demonstrated that S1P activation of $S1P_1$ results in Akt-dependent phosphorylation of eNOS and increased NO [50,51,53,75]. This suggests that $S1P_1$ activation may affect vasodilatation in conjunction with VEGF.

10. Endothelial cell migration, proliferation and morphogenesis

Directional endothelial cell motility is driven by a number of chemoattractants that bind GPCRs (interleukin-8 and fMLP) or growth factors, such as VEGF and fibroblast growth factor (FGF) [72,76,77]. Several studies have shown that $S1P_1$ is a critical regulator of endothelial cell migration and proliferation [50,51,55,73,78–80]. However, inhibition of NO production had no effect on S1P-induced endothelial cell chemotaxis, whereas VEGF-dependent chemotaxis was blocked [51].

$S1P_1$ activation also regulates many of the components that are involved in morphogenesis. S1P stimulation of $S1P_1$ and $S1P_3$ expressed in HUVECs results in activation of $\alpha_v\beta_3$- and β_1-containing integrins [54]. In addition to regulating cell spreading and migration, antagonists of $\alpha_v\beta_3$ and β_1-containing integrins inhibited S1P-induced endothelial cell morphogenesis in a three-dimensional fibrin matrix [54]. Activation of $S1P_1$ and $S1P_3$ also activate Rac- and Rho-dependent adherens junction assembly and cytoskeletal rearrangement that ultimately result in differentiation into capillary-like networks [57]. Rac and Rho are involved in S1P-stimulated translocation of VE-cadherin and β-catenin to cell-cell junctions [57]. Interestingly, in contrast to the action of S1P, VEGF disrupts adherens junctions [36,57].

11. Maturation of neovasculature

Once the neovasculature has been formed, endothelial cells must deposit a new basement membrane and recruit surrounding vessel layers composed of mural cells, such as pericytes in small vessels and smooth muscle cells in large vessels [76,77,81]. Recruitment of mural cells is largely dependent upon the synthesis and secretion of PDGF within endothelial cells [72]. On endothelial cell-mural cell contact, a latent form of transforming growth factor-β (TGF-β), produced by both endothelium and mural cells, is activated in a plasmin-mediated process [76] and induces changes in myofibroblasts and pericytes, leading to the formation of a quiescent vessel, ECM production and maintenance of growth control [76].

Studies on $S1P_1$ knockout mice showed that it is essential for vascular maturation as its gene disruption resulted in impaired vascular maturation due to the failure of mural cells to migrate to arteries and capillaries to reinforce them [47]. In fact, although $S1P_1$ null embryos died in utero due to massive hemorrhage, they exhibited normal vasculogenesis and a substantially normal blood vessel network, yet were severely impaired in the recruitment of smooth muscle cells and pericytes to the vessel walls and this was attributed to their defective migration [47]. Extracellular S1P can directly stimulate $S1P_1$ on vascular smooth muscle cells (VSMCs), facilitating their migration to vessel walls or, alternatively, can stimulate $S1P_1$ expressed in endothelial cells that in turn may recruit VSMCs [47]. Recent studies have demonstrated that the effect of $S1P_1$ on vascular maturation can be attributed to the cross-talk between $S1P_1$ and PDGF receptor signaling [55,80] (Fig. 2). Cell migration toward PDGF, which stimulates SPHK and increases intracellular S1P, was dependent upon $S1P_1$ expression in a number of cell types, including HEK 293 cells, human aortic smooth muscle cells (ASMCs) and MEFs [55]. It was therefore suggested that spatially and temporally localized generation of S1P by activation of SPHK in response to PDGF results in restricted activation of $S1P_1$ that in turn activates Rac, resulting in an increase in cell

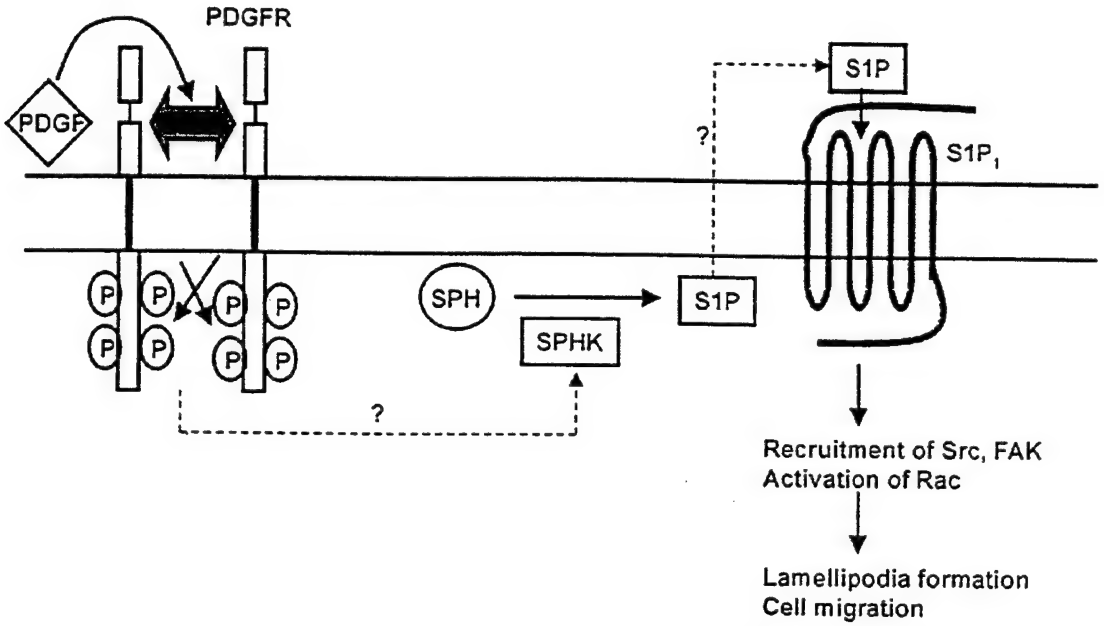

Fig. 2. Cross-talk between PDGFR and $S1P_1$ and its role in cell migration. Cell migration towards PDGF, which stimulates SPHK and increases intracellular S1P, has been shown to be dependent upon $S1P_1$ expression. PDGF-dependent-generation of S1P by activation of SPHK results in $S1P_1$-dependent activation of Rac, leading to cell migration towards PDGF.

motility [55]. Moreover, PDGF-induced cytoskeletal rearrangements, lamellipodia extensions and cell motility are abrogated in $S1P_1$ null fibroblasts [80]. Also, PDGF-induced focal adhesion formation and activation of FAK, Src and SAPK 2 were disregulated in the absence of $S1P_1$ [80]. However, $S1P_1$ was not involved in mitogenicity and survival effects induced by S1P or PDGF [80]. Hence, it was suggested that $S1P_1$ acted as an integrator linking the PDGFR to lamellipodia extension and cell migration [55,80].

12. The role of S1PR cross-talk in S1P signalling

As outlined above, many of the effects induced by extracellular S1P can be attributed to cross-talk between different receptors. For instance, activation of $S1P_1$ and $S1P_3$ is required for the activation of Rho and integrin in HUVECs, yet activation of Rac only requires $S1P_1$ [54,57]. Also, proliferation of human aortic endothelial cells requires both $S1P_1$ and $S1P_3$ signaling [73]. Cross-talk between $S1P_1$ and $S1P_2$ is also involved in the activation of ERK1/2 in C6 glioma cells [44]. Additionally, cross-talk has also been described between $S1P_1$ and the PDGFR, suggesting that further cross-talk mechanisms may exist between other receptor family members [55,80]. In fact, it has recently been demonstrated that $S1P_1$ can be phosphorylated in an agonist-independent manner *via* the activation of PKC [46] as well as in an agonist-dependent manner *via* GRK2 activation (Fig. 3). Hence, it is possible that $S1P_1$ may also be regulated by other receptor signaling mechanisms through PKC activation.

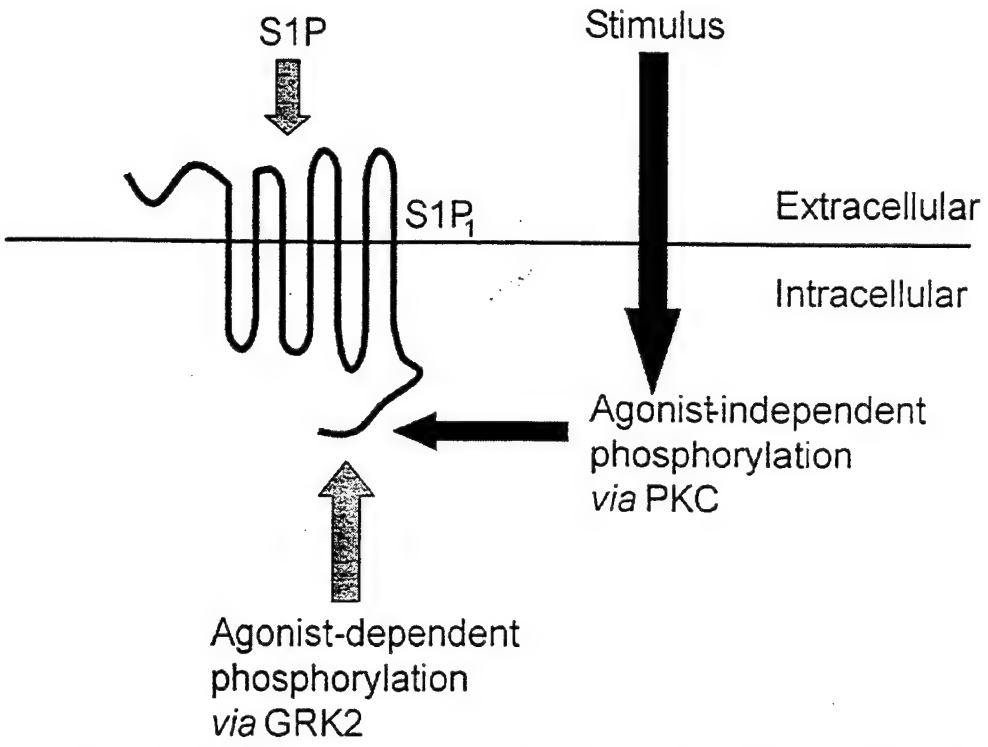

Fig. 3. Potential involvement of PKC in S1P-mediated phosphorylation of $S1P_1$. Binding of S1P to $S1P_1$ can lead to activation of phosphorylation of $S1P_1$. $S1P_1$ can also be phosphorylated in an agonist-independent manner *via* the activation of PKC. See text for more information.

The exact mechanisms involved in such cross-talk mechanisms constitute an interesting and rapidly growing aspect of S1PR research. One intriguing possibility is that receptor cross-talk is influenced by receptor dimerization. A recent study has demonstrated that a high degree of dimerization exists within the S1PR family [82]. For example, $S1P_1$, $S1P_2$ and $S1P_3$ have been shown to exist as monomers and as dimers independent of agonist activation [82]. Interestingly, dimerization has also been shown between $S1P_1$ and $S1P_3$, $S1P_1$ and $S1P_2$ and also $S1P_1$ and $S1P_2$ [82]. The implications of S1PR dimerization in terms of signaling are unknown. However, the possibility remains that many of the effects requiring more than one S1PR may be influenced by receptor dimerization. It is also possible that S1PRs may form complexes with other receptor subtypes. It was previously shown that co-stimulation of airway smooth muscle cells with S1P and PDGF elicits stronger p42/p44 MAPK activation than each agonist alone [83]. It was subsequently demonstrated that the PDGFR can also form a tethered complex with $S1P_1$ [84]. This complex enables the PDGFR to induce more efficient tyrosine phosphorylation of $G_{\alpha i}$ released upon stimulation of $S1P_1$ and that tyrosine phosphorylation of $G_{\alpha i}$ was required for PDGF and S1P to stimulate the p42/p44 MAPK pathway [84]. Interestingly, stimulation of the p42/p44 MAPK pathway promotes endothelial cell entry into the cell cycle, and induces transcription of VEGF, all of which are important in the process of angiogenesis [85].

13. Future directions and conclusions

Within the past few years there has been much progress in understanding the signaling properties and functions of the different S1PRs. Many studies point to an important role of $S1P_1$ in vascular maturation and angiogenesis . Much less is still known of the physiological and pathological functions of the other S1PRs. A better understanding of S1P signaling pathways, whether intra- or extracellular, should be useful in identifying targets for the development of therapeutics for a number of disease states. For example, there is much interest in the development of $S1P_1$ antagonists and/or SPHK inhibitors for the treatment of cancer since S1P plays such an important role regulating endothelial cell proliferation, survival, migration and vascularization, all critical processes in cancer progression. Development of specific S1PR agonists and antagonists should allow for a more accurate delineation of the effects of these receptors and provide potentially useful new therapeutics specifically targeting this novel sphingolipid metabolite.

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CHAPTER 163

Sphingosine-1-Phosphate Receptors

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Introduction

The endothelial differentiation gene (EDG) family of G-protein coupled receptors (GPCRs) comprises high-affinity receptors for the lysophospholipids, lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P) [1,2]. The homologous EDG receptors are clearly divided into two classes: three that bind LPA (EDG-2/LPA_1, EDG-4/LPA_2, EDG-7/LPA_3) and five that bind S1P (EDG-1/$S1P_1$, EDG-3/$S1P_3$, EDG-5/$S1P_2$, EDG-6/$S1P_4$, EDG-8/$S1P_5$). Molecular modeling and targeted mutagenesis have shown that S1PRs and LPARs use very similar motifs for binding of ligands, with one amino acid primarily determining the difference in specificity [3]. As several excellent reviews have recently appeared on LPARs and our studies have concentrated on dissecting molecular signaling pathways regulated by S1P [4,5], we have focused in this chapter on lipid signaling to and through S1PRs.

S1P is formed by sphingosine kinase (SphK), of which there are two known mammalian isoforms (for review, see [6]). SphKs are evolutionarily conserved and catalyze the ATP-dependent phosphorylation of the primary hydroxyl of sphingosine, the common backbone of mammalian sphingolipids. S1P is an interesting molecule that is an intercellular messenger and an intracellular second messenger [7]. This greatly complicates interpretation of results when adding exogenous S1P to cells: Is the response observed due to cell surface receptors, effects on intracellular targets, or both? A preponderance of studies have indicated that many of the biological effects of S1P are mediated by specific S1PRs and the lack of confirmed intracellular targets appears to bolster these claims. However, others have suggested that certain results are better explained by receptor-independent intracellular effects of S1P. First, the well-known S1PRs typically have K_ds in the 2–30 nM range [2,8], whereas effects of S1P on growth and suppression of apoptosis usually require micromolar concentrations [9]. In addition, dihydrosphingosine-1-phosphate (dhS1P), which has the same structure as S1P but only lacks the 4,5-*trans* double bond, binds to and activates all of the S1PRs. However, dhS1P does not mimic the effects of S1P on growth and survival [10], thus suggesting that these effects are likely to be mediated by intracellular actions of S1P.

The S1PRs

S1P was identified as the natural high affinity ligand of $S1P_1$ [2], which was shown to be highly specific, only binding S1P and dhS1P [11,12]. $S1P_1$ is coupled to $G_{\alpha i}$ and $G_{\alpha o}$ [13] but not $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha 12/13}$ [14]. Thus, pertussis toxin, which inhibits $G_{\alpha i/o}$ proteins, is a useful tool for dissecting signaling through $S1P_1$. *$s1p_1$* deleted mice died *in utero* between E12.5 and E14.5 due to massive hemorrhaging [15]. Although vasculogenesis and angiogenesis are normal in the *$s1p_1$*-/- mice, vascular smooth muscle cells failed to completely surround and seal the vasculature, thereby leading to hemorrhage. On a cellular level, the defect was linked to an inability of $S1P_1$ null fibroblasts to migrate toward S1P, likely due to dysfunctional Rac activation, and indicated the important role of S1P/$S1P_1$ signaling in motility.

$S1P_2$ is unique in being the only one of the S1PRs with a significantly poorer affinity for dhS1P than S1P [16]. $S1P_2$ has a wide tissue distribution [17] and a K_d for S1P of 20–30 nM [11]. In addition to $G_{i/o}$, $S1P_2$ couples to $G_{\alpha q}$ and $G_{\alpha 12/13}$ [14]. $S1P_2$ has been linked to increases in cAMP levels and thus may couple weakly to $G_{\alpha s}$ in some cell types depending on the pattern of expression of both GPCRs and

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G proteins [18]. $S1P_2$ regulates diverse signaling pathways, including calcium mobilization, stimulation of NF-κB, and inhibition of Rac-dependent cell migration in certain cell types [19,20]

$S1P_2$ has also been knocked out in mice [21] and in contrast to *s1p1-/-* mice, these mice have no obvious anatomical or physiological phenotypes. It is interesting that mammalian $S1P_2$ is highly homologous to the zebrafish gene *miles apart* [22]. Two inactivating mutations in *miles apart* prevent the normal migration of heart primordia, thus resulting in abnormal cardiac development. The heart precursor cells from the *miles apart* mutants migrated normally when transplanted into wild-type embryos, but wild-type cells failed to migrate in mutant embryos, a result that suggests that the zebrafish $S1P_2$ homologue is required for generating a migration-permissive environment.

$S1P_3$ was shown to be activated by S1P [23] with a K_d of 20–30 nM [11,12] and to couple to $G_{\alpha i/o}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$, but not $G_{\alpha s}$ [14]. $S1P_3$-null mice have been generated and also have no obvious phenotype [24]. $S1P_3$ has been linked to many signaling pathways, including calcium mobilization, stimulation of NF-κB, and NO production [25,26].

The two remaining S1PRs have a more narrow tissue distribution. $S1P_4$ is expressed almost exclusively in lymphoid and hematopoietic cells, as well as in the lung [27]. $S1P_4$ has a K_d for S1P of 12–63 nM, as determined by different groups [28,29], and couples to $G_{\alpha i/o}$. The final S1PR, $S1P_5$, previously named EDG-8 and *nrg-1*, is expressed predominantly in the central nervous system and to a lesser extent in lymphoid tissue [8,30]. $S1P_5$ couples to $G_{\alpha i/o}$ and $G_{\alpha 12/13}$, but not to $G_{\alpha s}$ or $G_{\alpha q}$ [31] and has a K_d for S1P of 2–6 nM [8,31].

S1P Signaling via S1PRs

Intriguing questions concerning lysolipid messengers are what regulates the levels of these amphipathic molecules and how do they get to their target cells? Platelets are known to store S1P and release it upon stimulation (reviewed in [32]). HUVECs and C6 glioma cells release S1P to the extracellular milieu [33,34]. Moreover, even when S1P release from cells is below detectable limits, co-culturing cells expressing $S1P_1$ with cells producing S1P due to overexpression of SphK induced activation of $S1P_1$ on adjacent as well as distant cells, thus indicating either that vanishingly small amounts of S1P are released or that it can be transferred from one cell to another by cell–cell interactions, or both [35]. Thus, S1P can act in an autocrine and/or paracrine manner. In support of this concept, the chemoattractant PDGF recruits SphK to the plasma membrane, where S1PRs are located, and especially to structures known as lamellipodia [36]. Given the importance of lamellipodia and S1PRs in chemotaxis, this finding suggests that S1P is produced and released from the cell in a spatially restricted manner, providing cells with a sense of direction. In addition, a recent report claims that type 1 SphK is secreted from cells in a catalytically active form and may catalyze the formation of S1P at or near the plasma membrane [33]. Further studies are necessary to confirm a role for extracellular SphK.

Transactivation of S1PRs

An intriguing aspect of the *s1p1-/-* phenotype is that it appears to be nearly identical to that of the PDGF-BB and PDGFR-β knockouts [15], as these embryos also die because of a vascular smooth muscle cell migration defect. Because PDGF stimulates SphK and increases S1P [10], it therefore appeared possible that $S1P_1$ and PDGF signaling pathways are linked. Indeed, embryonic fibroblasts from *s1p1-/-* mice, in contrast to wild-type cells, failed to migrate toward both S1P and PDGF [35]. Moreover, enforced expression of $S1P_1$ in HEK 293 cells, which express low basal levels of $S1P_1$, increased their ability to migrate toward PDGF, and antisense ablation of $S1P_1$ significantly inhibited migration toward PDGF [36]. A specific inhibitor of SphK also blocked PDGF-induced motility. Taken together, these results suggest a transactivation pathway linking PDGF though SphK to the autocrine and/or paracrine release of S1P that then stimulates $S1P_1$ to regulate motility. Furthermore, it was independently shown that $S1P_1$ potentiated the response to PDGF in HEK 293 cells overexpressing PDGFR [37]. However, in this case, these effects appeared to be independent of SphK, and it was suggested that PDGFR and $S1P_1$ were tethered in a complex that was activated independently of S1P.

Downstream Signaling from S1PRs

Because the S1PRs are coupled to heterotrimeric G proteins, the types of signals transduced are many and varied, depending on the specific isoforms of G_α and $G_{\beta\gamma}$ that are present. Thus, signals linked to a S1PR in one cell type may not be linked in the same manner in a second cell type. For example, transfection with $S1P_1$ increases S1P-induced calcium mobilization in CHO cells [38] but not in COS-7 cells [39]. Determining which specific S1PR is involved in a particular response is difficult because most cells express multiple S1PRs. To date, S1PR specific agonists or antagonists have not been developed. Thus, to elucidate the role of a particular S1PR, either transfection of receptor negative or knockout cells or antisense approaches have been used. Given the diversity of GPCR signaling, it is not surprising that results from these experiments demonstrate that S1PRs control the major lipid-mediated signaling pathways, as discussed below.

Phospholipase C. Many of the responses linked to S1PR signaling involve increases in intracellular calcium. Generation of the second messenger inositol trisphosphate (IP_3) by activation of phospholipase C (PLC) is the major pathway leading to intracellular calcium increases. CHO cells transfected with $S1P_1$, $S1P_2$, $S1P_3$, or $S1P_4$, but not vector controls, had increased IP_3 production and calcium release in an

S1P-dependent manner [28]. In contrast, in Jurkat T cells, $S1P_2$ and $S1P_3$, but not $S1P_1$, elicited IP_3-mediated calcium responses [25]. On a more physiological level, in HUVECs, which express $S1P_1$, and to a lesser extent $S1P_3$, S1P stimulated nitric oxide (NO) production by calcium-dependent epithelial nitric oxide synthase (eNOS) [40]. NO production was blocked by the PLC inhibitor U73122, the calcium chelator BAPTA-AM, and antisense oligonucleotides to $S1P_1$ or $S1P_3$, thus demonstrating a role for both S1PRs in activation of PLC. Furthermore, fibroblasts from $S1P_3$-null mice, but not littermate controls, failed to activate PLC upon S1P addition [24].

Phospholipase D. Another important lipid second messenger is phosphatidic acid (PA), which is generated by activation of phospholipase D (PLD). Overexpression of $S1P_1$ in HEK 293 or NIH 3T3 cells did not result in activation of PLD [9]. However, in C2C12 skeletal muscle cells, S1P stimulated PLD via either $S1P_1$, $S1P_2$, or $S1P_3$ in a pertussis toxin–sensitive manner [41]. Transfection of either $S1P_1$ or $S1P_2$ in C6 glioma cells conferred S1P-dependent PLD stimulation and PA formation [42]. $S1P_3$ also induced production of PA, specifically through activation of PLD2 in CHO cells [43].

Phosphatidylinositol-3-kinase. Activation of phosphatidylinositol-3-kinase (PI3K) promotes cell survival, cytoskeletal remodeling, and vesicular trafficking [44]. PI3K also promotes activation of the protein kinase Akt in two ways: translocation of Akt to the membrane by binding phosphatidylinositol-3,4-bisphosphate and activation of phosphoinositide-dependent kinases, which phosphorylate and activate Akt (reviewed in [45]). Though the S1PR(s) involved were not identified, S1P induced chemotaxis and angiogenesis of endothelial cells both *in vivo* and *in vitro* in a PI3K- and Akt-dependent manner [46,47]. $S1P_1$ transiently transfected in COS-7 cells led to activation of Akt, which was inhibited by the PI3K inhibitor wortmannin [48]. Further work from this group implicated $G_{\beta\gamma}$ stimulation of the PI3Kβ isoforms in S1P-dependent signaling to PI3K [49]. On a more physiological level, ventricular cardiomyocyte hypertrophy induced by S1P was inhibited by both wortmannin and by $S1P_1$ antibody [50]. $S1P_1$, $S1P_2$, and $S1P_3$ transfected into CHO cells each activated PI3K in response to S1P [43,51]. It is interesting that in this system, $S1P_1$ and $S1P_3$ promoted S1P-induced chemotaxis, while $S1P_2$ inhibited it.

Sphingosine Kinase. S1P has been demonstrated to release calcium from non-IP_3 releasable microsomal stores, though the intracellular receptor(s) are unknown [52–54]. Meyer zu Heringdorf and colleagues demonstrated that HEK 293 cells endogenously expressing $S1P_1$, $S1P_2$, and $S1P_3$ mobilized calcium in response to S1P [55]. However, in these cells, PLC was not activated and there was no measurable production of IP_3. What is especially interesting, they found that S1P stimulated SphK and S1P production, and the increase in S1P levels, as well as calcium release, was reduced by inhibitors of SphK. S1P production was also completely blocked by pertussis toxin, indicating the involvement of G_i-linked GPCRs in the process. Thus, the remarkable observation was made that extracellular S1P regulates intracellular S1P formation [55].

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APPENDIX 4

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Glycosphingolipids and cell death

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Sphingolipids have been implicated in various cellular processes including growth, cell-cell or ligand-receptor interactions, and differentiation. In addition to their importance as reservoirs of metabolites with important signaling properties, sphingolipids also help provide structural order to plasma membrane lipids and proteins within the bilayer. Glycosylated sphingolipids, and sphingomyelin in particular, are involved in the formation of lipid rafts. Although it is well accepted that ceramide, the backbone of all sphingolipids, plays a critical role in apoptosis, less is known about the biological functions of glycosphingolipids. This review summarizes current knowledge of the involvement glycosphingolipids in cell death and in other pathological processes and diseases.
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Keywords: **glycosphingolipids, apoptosis, GD3, glucosylceramide**

Introduction

Glycosphingolipids (GSL) are lipid components of membranes that are important for the proper development of vertebrates. They are involved in multiple processes, including cell type specific adhesion, cell-cell interaction, embryogenesis, and development and differentiation of neuronal cells and leukocytes [1]. GSL can also serve as binding sites for several viruses, bacteria, and bacterial toxins [2]. Different tissues display different GSL patterns on the cell surface which can be dramatically altered during development [1]. A further modulation can be seen during pathological processes such as tumor development. GM3/GD3, for example, is a melanoma-associated antigen involved in metastasis [3–5]. On the other hand, glucosylceramide (GlcCer) expression is associated with multidrug resistance in many cancer cells [6–8].

GSL are predominantly located at the plasma membrane and the early endosomes of the Golgi complex. In the plasma membrane, it has recently been shown that sphingolipid-derived molecules aggregate and form a less fluid and more ordered phase, referred to as membrane rafts, which are formed in the Golgi compartment and targeted to the plasma membrane. Rafts are considered to be small, mobile lateral assemblies of sphingolipids, particularly enriched in sphingomyelin and cholesterol, but also containing ceramide and GPI-anchored proteins. They have important roles in concentrating and modulating specific signaling molecules, such as Src-tyrosine kinase family members, growth receptors, and death receptors [9–12]. The role of rafts will not be discussed here as it has been the subject of recent excellent reviews [9,13]. Less complex sphingolipid-derived molecules, including ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate (S1P), are known signaling molecules in diverse receptor and non-receptor-mediated signaling pathways. These bioactive lipid mediators are formed as a result of stimuli-induced metabolism of complex sphingolipids. Ceramide has mainly been implicated in signaling pathways leading to suppression of growth, cellular senescence, differentiation, and apoptosis, whereas ceramide-1-phosphate mediates cell survival and is involved in synaptic vesicular fusion in neuronal cells, as well as neutrophil phagolysosome formation [14]. S1P has many biological actions and, importantly, acts counter to ceramide to mediate cell growth and survival, as well as influencing directed cell movement [15,16]. The biological effects of sphingosine may vary among cell types but it has been associated with negative effects on cell growth and survival and has been implicated as an inhibitor of protein kinase C and other protein kinases [17,18].

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Biosynthesis and structure of glycosphingolipids

The *de novo* biosynthesis of GSL is initiated at the cytosolic surface of the endoplasmic reticulum (ER) by the condensation of L-serine and palmitoyl coenzyme A to form 3-ketosphinganine catalyzed by serine palmitoyltransferase (SPT), a pyridoxal phosphate-dependent enzyme [19,20]. SPT has lower activity

mitochondria. ROS have been implicated in the initiation phase as well as in the execution phase of the apoptotic program, depending on cell type. Also, addition of ROS or depletion of endogenous antioxidants induces apoptosis that can be reversed by exogenous addition of antioxidants. The detailed mechanisms by which ROS function in cell death are not yet clear, but they are likely to be involved in activation of executionary caspases. In TNF-α-resistant heparocytes, it was demonstrated that TNF-α was still able to induce synthesis of GD3. Only after depletion of mitochondrial glutathione, which is crucial for the maintenance of the cellular redox state, were cells sensitized to TNF-α- or GD3-mediated apoptosis [52]. This study underlines the importance of oxidative stress in TNF-α-mediated apoptosis in hepatocytes. By using inhibitors or antioxidants, it was shown that GD3 interacts with complex III of the mitochondrial electron transport chain causing an oxidative burst that precedes mitochondrial swelling. Cytochrome c and apoptosis inducing factor (AIF) are subsequently released, leading to activation of the caspase cascade and eventual DNA fragmentation [50,51]. *In vitro* studies performed with isolated mitochondria revealed that short-chain ceramides and glycosphingolipids, such as GlcCer, LacCer, GD1a, and GM1, are able to mimic GD3, while sphingosine and sphinganine failed to do so. Apparently, the *N*-acylsphingosine (ceramide) moiety is required for interaction of GD3 with the mitochondria rather than the carbohydrate component [50]. However, other groups showed that GD3-mediated effects on isolated mitochondria are very specific and could not be mimicked by C_2-ceramide, GM1, GM3, GD1a, or GT1b [53,54]. Future studies are needed to clarify this discrepancy and to determine the minimal structural requirement that enables GD3 to interact with and recruit mitochondria to the apoptotic signal transduction pathway.

GD3 can directly activate $\Delta\Psi_m$ independently of Ca^{2+}, although Ca^{2+} has been shown to act synergistically with GD3 [53]. It has been suggested that the effects of GD3 on mitochondria are mediated by the opening of the mitochondrial permeability transition pore (MTP), rather than by inhibition of the respiratory complex, as GD3-mediated effects could be prevented with the MTP blocker, cyclosporin A [54]. The MTP is a conductance channel formed by several different proteins, which is inserted into the mitochondrial membrane [55].

Bcl-2 is a proto-oncogene known to suppress cell death by diverse stimuli [55]. One mechanism by which Bcl-2 protects cells is suppression of the formation of ROS by acting as an antioxidant [55,56]. Because several studies have shown that ROS production in the mitochondria is a key target for apoptogenic GD3, it is conceivable that Bcl-2 might be able to modulate this pathway as well. Indeed, in T cell lymphoma CEM cells stably overexpressing Bcl-2, GD3 failed to induce mitochondrial changes or release of cytochrome c, AIF and activate caspase-9 [51]. Similar observations were made in oligodendrocytes where GD3-induced increase in $\Delta\Psi_m$ and cytochrome c release could be partially blocked by enforced Bcl-2 expression [57]. How Bcl-2 blocks GD3-induced cell death is not known yet. Several models have been proposed to explain how Bcl-2 might exert its anti-apoptotic function. It could prevent pore formation induced by other pro-apoptotic Bcl-2 family members, such as Bax/Bak, via increased heterodimerization of these proteins, or it could inhibit the opening of the MTP [58,59]. Moreover, Bcl-2 family proteins appears to regulate voltage-dependent anion channel (VDAC) function [60,61]. Further studies are need to identify the relevant GD3 targets which are under Bcl-2 control. However, pretreatment of isolated mitochondria with cyclosporin completely suppressed GD3-induced swelling and release of apoptogenic factors, indicating that GD3 acts at the level of the MTP. Whether this is due to a direct interaction with any of the MTP components remains to be established [51].

Ceramide can be generated from degradation of sphingomyelin by either acidic sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase). Alternatively, ceramide generated by *de novo* synthesis has also recently been implicated in apoptosis [62]. Furthermore, it has been reported that ceramide generated by aSMase, and not nSMase, is involved in Fas and TNF-α signaling pathways activated in GD3 mediated cell death even though nSMase is active and contributes to the increase in ceramide in human colon cells [63]. These results were further confirmed with Niemann-Pick-derived lymphoblastoid cells that are devoid of aSMase but display normal nSMase activity [64]. In these cells, Fas failed to initiate the apoptotic program. Reconstitution of aSMase activity or addition of exogenous aSMase, however, caused GD3 accumulation and efficiently triggered the apoptotic program after Fas cross-linking or γ-irradiation.

Gangliosides are distributed predominantly on the plasma membrane and in the early Golgi compartment where they are synthesized. GD3 synthase (α2,8-sialyltransferase), which resides in the Golgi, adds a second sialic acid to GM3 to produce GD3. Just as is the case with ceramide, there seems to be a dichotomy in the signaling properties between newly synthesized GD3 and GD3 formed from degradation of other complex gangliosides. It appears in this case that newly-synthesized GD3 is involved in regulating apoptosis [65]. The question arises as to how newly formed GD3 is targeted to mitochondria where it executes its function in cell death. Different pathways might come into play. First, mitochondria might be in close physical contact with the ER/early Golgi to form a functionally interconnected network which has been described recently [66–68]. Second, and more likely, GD3 might be redistributed to mitochondria by actin-dependent endosomal vesicles. Indeed, disruption of actin cytoskeletal organization prevents release of GD3 from plasma membrane and co-localization with mitochondria [65]. Also, GD3 was shown to co-localize and associate with the actin cytoskeletal protein ezrin upon Fas cross-linking [69]. Moreover, pretreatment of cells with inhibitors of vesicular transport, such as monensin or mannose-6-phosphate, abolished localization of GD3 with mitochondria in hepatocytes treated with TNF-α [65]. Trafficking of GD3 was monitored over time and co-localization of GD3 was seen with markers specific for

plasma membrane, early endosomes, late endosomes, and finally with mitochondria [65]. GD3 ganglioside on the plasma membrane is localized, most likely, in specialized rafts, known as caveolae, where it can be internalized through endocytosis and trafficked to mitochondria. Co-localization of GD3 with caveolin-1 has been described previously [70].

As mentioned above, only ceramide generated in specific compartments, such as mitochondria or at the plasma membrane, has been shown to be involved in programmed cell death [47,71,72]. Furthermore, depending on cell type and/or agonist, aSMase and/or nSMase contribute to ceramide generation. Cells derived from aSMase null mice are defective in Fas-, radiation-, and TNF-α-induced cell death [63,64,73,74]. aSMase is active mainly in acidic compartments, such as recycling endosomes, and soluble aSMase is taken up by endocytosis and transported to acidic compartments. Membrane-bound forms of aSMase have also been detected in caveolae microdomains enriched in sphingomyelin that can be activated by various stimuli resulting in formation of ceramide [75,76]. Translocation of aSMase from intracellular compartments to plasma membrane rafts has been demonstrated after Fas stimulation [77].

Two different cytoplasmic domains have been described in the 55 kDa TNF receptor. One domain is able to activate nSMase and the other activates aSMase with no apparent crosstalk between them. The aSMase activation domain resides in the so-called death domain of the TNF receptor responsible for the cytotoxicity of TNF-α [78]. A phosphatidylcholine-specific phospholipase C activity was also required for aSMase activation [79,80].

It is assumed that intracellular ceramide concentrations regulate sphingolipid and glycosphingolipid metabolism; and, hence, ceramide should be targeted to the Golgi complex. Because aSMase has been shown to reside in caveolae, decreased sphingomyelin and concomitant ceramide production can lead to structural changes of the plasma membrane which can somehow stimulate endocytosis and trafficking of ceramide to the ER and Golgi, thereby enhancing GD3 synthesis. This might also explain why ceramide generated via aSMase, but not nSMase, is able to activate *de novo* GD3 synthesis.

Glycosphingolipids with anti-apoptotic properties

Difficulties in effective chemotherapy correlate with defective activation of programmed cell death on several distinct levels in many types of tumors [81]. Chemotherapeutic agents often exert some of their effects through generation of ceramide even though their mechanisms of action might differ.

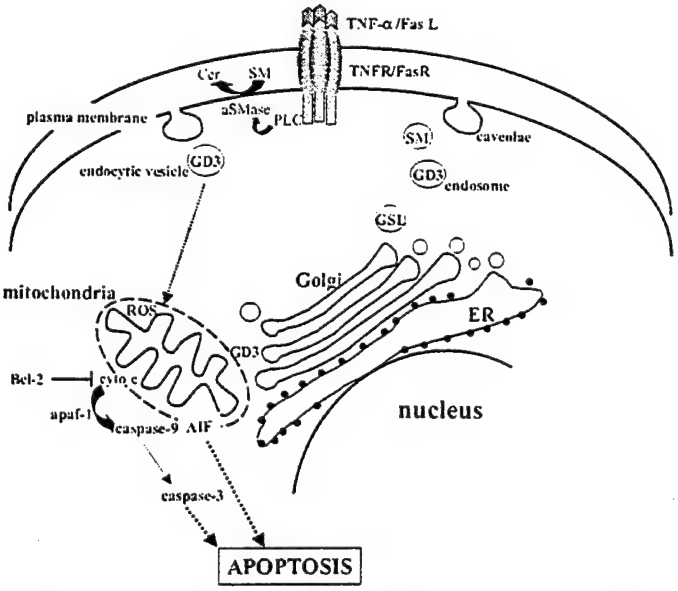

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Figure 2. Signaling pathway of GD3-induced apoptosis. After oligomerization of the Fas or TNF-α receptors, aSMase is activated in a PC-PLC-dependent manner. Ceramide accumulates and activates GD3 synthesis. GD3 is targeted from the plasma membrane by vesicle transport or alternatively by physical redistribution from the Golgi to the mitochondria. There, GD3 perturbs the mitochondrial membrane leading to release of cytochrome c and AIF and caspase-9 activation, which activate the execution phase of the apoptotic program leading to demise of the cell.

Some stimulate *de novo* ceramide synthesis whereas others induce sphingomyelin hydrolysis or block ceramide degradation. Multidrug resistance, defined as cross-resistance to a variety of chemotherapeutic substances, is a common phenomenon in the treatment of various cancers. Besides accelerated removal of the drug (i.e. enhanced drug efflux via the P-glycoprotein pump), their intracellular effects may be altered [82,83]. It is also possible that multidrug resistance could result from modulation of ceramide metabolism whereby ceramide accumulation is prevented or it is converted to less toxic molecules [6,7,84]. Yet another way to keep endogenous ceramide low is by GlcCer synthase catalyzed conversion to GlcCer which has been shown to have growth stimulatory and anti-apoptotic effects [85]. Studies with exogenous administration of GlcCer revealed that it is able to stimulate growth of keratinocytes even in aged murine epidermis where epidermal growth is normally reduced [86]. Furthermore, GlcCer is consistently increased in several multidrug-resistant cancer cell lines [83,87]. In this regard, it was demonstrated that some sensitive cells acquire drug resistance by overexpressing GlcCer synthase [88]. Conversely, blocking glycosylation of ceramide with different agents, such as verapamil, tamoxifen, cyclosporin A or PDMP, in multidrug resistant MCF-7 breast cancer cells, sensitized them to adriamycin [89]. In addition, GlcCer synthase antisense RNA rendered otherwise resistant cells sensitive to drug treatment [88–91]. Reduced tumorigenicity and metastatic potential of melanoma cells was also observed *in vivo* with GlcCer synthase antisense RNA [92].

Another ganglioside that has been implicated in protection of cells from apoptosis is the monosialylganglioside GM1. GM1 has been shown to prevent apoptotic cell death in growth factor-deprived neuronal PC12 cells [93]. GM1 acts by promoting nerve growth factor (NGF)-induced TrkA dimerization. It has also been demonstrated that NGF signaling can activate sphingosine kinase to form S1P that acts as a pro-survival signal [93,94]. Similar results were obtained in a study conducted in rat heart fibroblasts where GM1 was shown to act like S1P and protect cells from C_2-ceramide or staurosporine-induced cell death [95]. It was also demonstrated in this study that GM1 enhanced S1P production by activating sphingosine kinase [95]. In a more physiologically relevant study, application of GM1 also protected the mouse heart from hypoxic cell death. Again, sphingosine kinase-dependent activation by protein kinase Cε was suggested [96]. These results have relevance to human physiology and there are ongoing clinical trials using GM1 ganglioside as a therapeutic agent for promoting nerve regeneration in Alzheimer's disease [97]. Furthermore, autoantibodies against various glycosphingolipids have been detected in patients with different neurological disorders, and have been suggested to play a critical role in development of diseases of the nervous system. In contrast, Le(y) antigen expression is correlated with apoptosis [98].

Gangliosides can also regulate cell signaling by altering growth factor receptor functions [99]. High GM3 ganglioside expression on keratinocytes has been correlated with inhibited cell growth and low expression has been reported in several hyperproliferative skin disorders, including psoriasis and squamous cell carcinoma [100,101] where programmed cell death is aberrant. Ganglioside GM3 was shown to interfere with binding of EGF and activation of its receptor which is required for proliferation [102].

Surprisingly, GM3 is also pro-apoptotic in certain types of cells, particularly in the presence of metastasis-suppressing gene product CD82 and its analogue CD9. It was shown that the malignancy-suppressing effect of CD82 or CD9 is based partially on cell motility inhibition and apoptosis induction promoted by concurrent GM3 synthesis and N-glycosylation [103]. GM3 in various colorectal carcinomas may also promote apoptosis, since enhancement of endogenous sialidase promotes tumor malignancy and metastasis through inhibition of Bcl-2 [104]. These dual actions of GM3 merit further study.

Significance of gangliosides in pathological processes

It has long been known that tumor cells display a different pattern of cell surface glycosphingolipids than corresponding untransformed cells [1,105]. Predominant expression of specific gangliosides, GD3, GM2, or GD2, has been observed on several types of tumor cells including melanoma, neuroblastoma, lymphoma, and ovarian cancer cells [105,106]. Thus, antibodies against specific gangliosides have received consideration as immunotherapeutic agents and clinical trials have been initiated [4,106–108].

Augmented GSL shedding, which is the release of cell surface components, is a characteristic of cancer cells. Shedding seems to be important for infiltration and metastasis of the tumor as well as for suppression of the immune system [109]. The underlying mechanism by which the released components evoke these biological effects is not yet fully understood. Gangliosides are among the main constituents of the released molecules. *In vitro*, and more importantly, *in vivo* effects of gangliosides shed from T cell lymphoma on bone marrow cells (BMC) have been documented [110]. These gangliosides not only impaired cell viability but also induced apoptosis of BMC. The shed gangliosides activated NF-κB and elevated expression of p53 and Bax, both of which have been described as components of pro-apoptotic signaling pathways. The apoptosis effects were ascribed to GD3 by investigations with antibodies against the major ganglioside species produced by T cell lymphomas. GD3 exogenously applied to BMC effectively induced apoptosis, further confirming this finding [110].

Another disease in which GD3 seem to be involved in is the progression of pathogenesis in Farber Disease, a lysosomal storage disorder which results from an acid ceramidase deficiency. As a consequence, ceramide accumulates in lysosomes leading to tissue damage, although the detailed mechanisms of tissue destruction are not well known. Histochemical analyses of tissues from affected patients revealed a high apoptotic rate

that correlated with concomitant elevated levels of GD3 and activated caspase-3 [111].

Conclusions

There is now abundant evidence documenting the importance of sphingolipid-derived signaling molecules. Although some sphingolipids have been well established as second messengers, i.e. ceramide, sphingosine and S1P, others await more detailed investigations. One difficulty in identifying the specific sphingolipid involved in a particular signaling pathway is their complex interconversion. For example, functions attributed to sphingosine might actually result from its conversion to S1P. Moreover, some effects of ceramide might result from its conversion to ceramide-1-phosphate, GlcCer, or even sphingosine and S1P. Also rapid degradation of gangliosides to ceramide has been described and this might confuse distinctions between ceramide and ganglioside-mediated effects [112]. As found for GM1, one GSL might also be able to stimulate the generation of another sphingolipid [95]. Since GD3 has mainly been associated with cell death and GM1 with survival, it will be very important to determine exactly what structural feature of these molecules is required for initiation of particular signaling pathways. This further emphasizes how important it is to elucidate the mechanism by which a molecule produces a certain biological response in order to design drugs for therapeutic applications.

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SPHINGOSINE-1-PHOSPHATE: AN ENIGMATIC SIGNALLING LIPID

Sarah Spiegel and Sheldon Milstien‡*

The evolutionarily conserved actions of the sphingolipid metabolite, sphingosine-1-phosphate (S1P), in yeast, plants and mammals have shown that it has important functions. In higher eukaryotes, S1P is the ligand for a family of five G-protein-coupled receptors. These S1P receptors are differentially expressed, coupled to various G proteins, and regulate angiogenesis, vascular maturation, cardiac development and immunity, and are important for directed cell movement.

Sphingosine-1-phosphate (S1P) is derived from sphingosine — the backbone of most sphingolipids — and it is now emerging as a vital lipid mediator. Sphingosine was named in 1884 after the Greek mythological creature, the Sphinx, because of its enigmatic nature[1]. More than a century later we are just beginning to unravel the riddle of S1P. S1P was originally considered to be formed merely as an intermediate in the detoxification of sphingosine, by its phosphorylation and subsequent degradation[2] (FIG. 1), but since the discovery that S1P regulates cell growth[3,4] and suppresses programmed cell death[5], there has been an explosion of important physiological and pathophysiological processes that are reported to be regulated by S1P in higher organisms. S1P and its homologous phosphorylated long-chain sphingoid bases have been detected in plants, worms, flies, slime mould and yeast, and they also regulate important biological responses even in these lower organisms, which further highlights the importance of S1P as a signalling molecule. The puzzle of how such a simple molecule can have such diverse roles has been resolved by the discovery that it belongs to a class of lipid mediators that function not only inside cells but also as ligands (agonists) for specific cell-surface receptors.

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S1P synthesis and degradation

As is the case for other signalling molecules, S1P levels in cells are low and tightly regulated in a spatial–temporal manner by the balance between its synthesis and degradation (FIG. 1). The activity of sphingosine kinase (SphK; BOX 1), which catalyses the ATP-dependent phosphorylation of sphingosine, is stimulated by many agonists, which indicates that this is a central regulating enzyme of S1P. The degradation of S1P is mediated by two different pathways: one is the reversible dephosphorylation back to sphingosine by specific S1P phosphatases (BOX 1); the second is the irreversible degradation by a pyridoxal phosphate-dependent S1P lyase (Spl) (BOX 1) to hexadecenal and phosphoethanolamine, which are subsequently reused for the biosynthesis of phosphatidylethanolamine (FIG. 1).

Sphingosine kinases. SphKs are a distinct and newly discovered class of lipid kinase that have five conserved domains and are evolutionarily conserved. They are expressed in humans, mice, yeast and plants, with homologues in worms and flies. Two mammalian isozymes, which are known as SphK1 and SphK2 (BOX 1), have been characterized[6]. These are located predominantly in the cytosol, although small amounts are associated with membranes. It is not yet clear whether these enzymes are present within organelles or whether they are loosely bound to cellular membranes, although in yeast, Lcb4 — the main enzyme that catalyses the formation of long-chain base phosphates — has been localized to the Golgi and late endosomes[7] and endoplasmic reticulum (ER)[8]. SphK1 and SphK2 have different kinetic properties, tissue distribution and temporal expression patterns during development, which indicates that they carry out distinct cellular functions and might be regulated differently. Their structural diversity and complex pattern of tissue

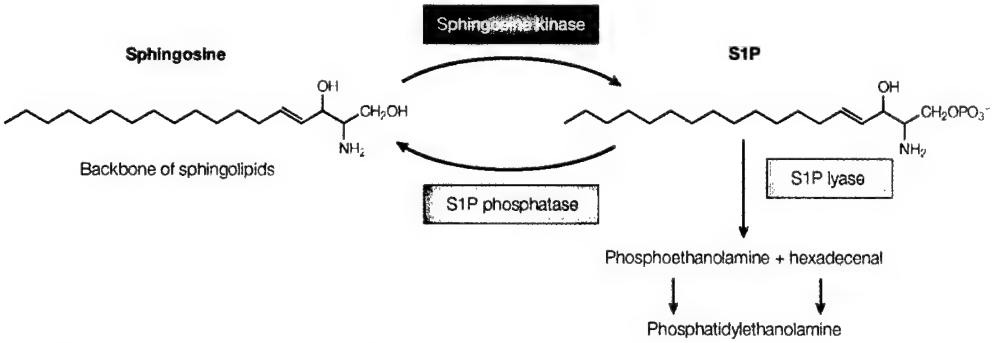


Figure 1 | **Formation and degradation of S1P.** The structures of sphingosine — the predominant sphingoid base of eukaryotic sphingolipids — and sphingosine-1-phosphate (S1P) are shown. Key metabolic enzymes for the formation and degradation of S1P are shown. Sphingosine kinases catalyse the formation of S1P from sphingosine. Conversely, two classes of enzyme — S1P phosphatases and S1P lyases — degrade S1P. The lyase products, hexadecenal and phosphoethanolamine, are reused for biosynthesis of phosphatidylethanolamine. See text and BOX 1 for further details.

expression is reminiscent of the large diacylglycerol kinase family, and it is probably not a coincidence that these two families share a high degree of homology in their catalytic domains. However, SphKs have a unique catalytic domain, which contains a consensus sequence of an ATP-binding site ($SGDGX_{(17-21)}K(R)$) that has some similarity to the highly conserved glycine-rich loop that is involved in binding the nucleotide in the catalytic site of many protein kinases[9].

S1P lyase. The main route of S1P degradation is through cleavage at the C2–C3 bond by Spl (BOX 1). The identification of the Spl gene in yeast[10], and subsequently in mammals and *Dictyostelium discoideum*, indicates that phosphorylated sphingoid base metabolism is a process that is conserved throughout evolution. Deletion studies have shown the importance of Spl and phosphorylated sphingoid bases in the regulation of global responses to nutrient deprivation in yeast[11]. Disruption of the *spl* gene in slime mould affects many stages of development, including the cytoskeletal architecture of aggregating cells, the ability to form migrating 'slugs' and terminal spore differentiation, which therefore implicates Spl in numerous processes in multicellular development[12]. It has been suggested that Spl is localized to the cytosolic face of the ER, at which all of the enzymes that are essential for sphingolipid biosynthesis reside (see below).

S1P phosphatase. Specific S1P phosphatases (SPPs) were first identified in yeast and shown to be important regulators of the heat-stress response[13–15]. On the basis of their homology with the yeast genes, the genes encoding two mammalian SPPs, SPP1 (REFS 16,17) and SPP2 (REF. 18), which only degrade phosphorylated sphingoid bases (BOX 1), were cloned. These SPPs belong to the family of magnesium-dependent, *N*-ethylmaleimide-insensitive type 2 lipid phosphate phosphohydrolases (LPPs)[19]. However, except for the conserved residues in three domains that are present in all LPPs[19], SPPs have little overall homology to other known LPPs. Moreover, whereas LPPs are present on the plasma membrane and function as so-called ecto-phosphatases to attenuate the actions of lysophosphatidic acid as an agonist of its cell-surface receptors[20], both SPP1 (REF. 21) and SPP2 (REF. 18), similar to their yeast counterparts[15], are localized to the ER[17,18].

Regulation of stress responses by S1P

S1P enhances growth and survival in diverse cell types[22]. By contrast, its precursors — ceramide (*N*-acyl sphingosine) and sphingosine — have generally been associated with growth arrest and cell death[23,24]. As these metabolites are interconvertible (FIG. 2), it has been proposed that it is not their absolute amounts, but rather their relative levels, that determine cell fate. The relevance of this 'sphingolipid rheostat' and its role in regulating cell fate has been borne out by the work of many researchers

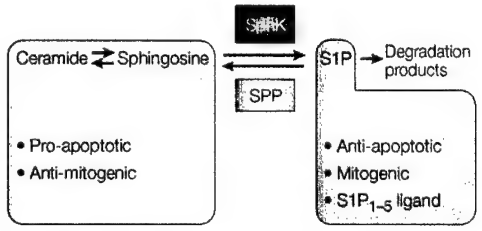


Figure 2 | **The sphingolipid rheostat: a conserved stress regulator.** Sphingosine-1-phosphate (S1P), sphingosine and ceramide are interconvertible sphingolipid metabolites. Many external stimuli have been shown to activate sphingosine kinases (SphKs) (BOX 3). SphKs converts sphingosine to S1P, thereby enhancing cell growth and survival. Conversely, S1P phosphatases (SPPs) remove the phosphate from S1P to form sphingosine, which is then *N*-acylated to form ceramide. Both ceramide and sphingosine have been associated with growth arrest and apoptosis. Whereas ceramide and sphingosine levels increase in response to many stress stimuli, suppression of apoptosis is associated with increases in S1P levels and decreases in ceramide (for reviews, see REFS 22,32,89). In addition, S1P regulates many processes by being able to interact with five specific cell-surface receptors (FIG. 4).

MITOCHONDRIAL OR INTRINSIC DEATH PATHWAY
The pathway that leads to death through the release, by stress-induced signals, of cytochrome *c* and other apoptogenic factors from the mitochondrial intermembrane space.

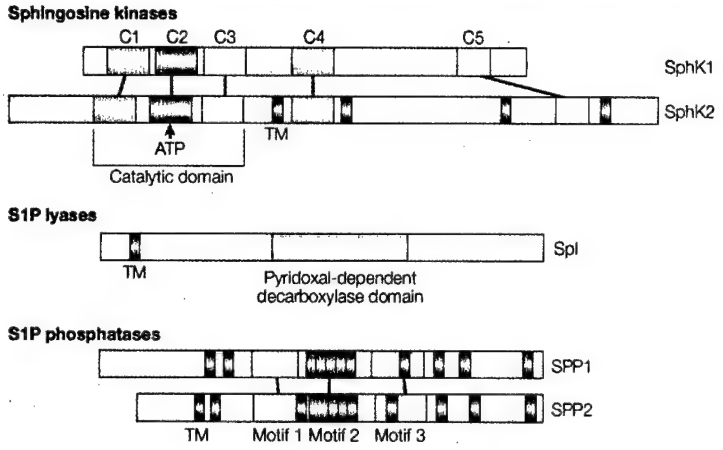
SPHINGOMYELINASE
An enzyme that catalyses the hydrolysis of sphingomyelin to ceramide (*N*-acylsphingosine) and choline phosphate.

CASPASES
A family of intracellular cysteine endopeptidases that have a crucial role in inflammation and mammalian apoptosis. They cleave proteins at specific aspartic acid residues.

using many different cell types and experimental manipulations.

A central finding of these studies is that SphKs and SPPs are essential regulators of the sphingolipid rheostat, and have converse effects on the pro-growth, anti-apoptotic messenger S1P versus the pro-apoptotic ceramide and sphingosine, and as a consequence, they have opposing effects on cell fate. For example, SphK1 and increased S1P levels enhance proliferation, expedite the G_1–S transition of the cell cycle and increase DNA synthesis[25]. SphK1 also promotes growth of cells in soft agar and tumour formation in mice, probably owing to its role in Ras and extracellular-signal regulated kinase (ERK)1/2 signalling[26–28]. In addition, SphK1 protects against apoptosis by inhibiting the MITOCHONDRIAL OR INTRINSIC DEATH PATHWAY, blocking the stress-activated protein kinase, Jun amino-terminal kinase (JNK)[25,29], and by activating nuclear factor κB (NF-κB)[30]. Deletion of both yeast SphK genes, *LCB4* and *LCB5*, showed that sphingosine is crucial for heat-induced G_0–G_1 arrest and that SphK removes the sphingosine block, allowing progression to S phase[31]. This indicates that there is a mechanism for cell-cycle control by the sphingolipid rheostat, which might be conserved in higher eukaryotes. Given the role of the sphingolipid rheostat in regulating growth and apoptosis, it is not surprising that sphingolipid metabolism is often found to be dysregulated in cancer, a disease that is characterized by enhanced cell growth, diminished cell death, or both.

Box 1 | Enzyme families that are important for regulating S1P levels



Sphingosine kinases

Two isoforms of sphingosine kinase (SphK) — Lcb4 and Lcb5 — were first cloned from *Saccharomyces cerevisiae* and show substantial sequence homology to mammalian SphK1 and SphK2 (REFS 80,81). SphK1 and SphK2 have five conserved domains (C1–C5) with the unique catalytic domain contained within C1–C3. The ATP-binding site ($SGDGX_{(17-21)}K(R)$) is present within C2. Hydropathy-plot analysis of the predicted amino-acid sequence of SphK1 does not indicate that any hydrophobic transmembrane regions (TM) are present, whereas SphK2 has four predicted transmembrane regions. Despite the overall homology between SphK1 and SphK2, the sequences of these two proteins diverge sufficiently to indicate that they might not have arisen from a simple gene-duplication event. Northern-blot analysis has shown that SphKs have different tissue distributions[6]: SphK1 expression is highest in lung and spleen, whereas SphK2 is most abundant in liver and heart. SphK1 expression is greatest at mouse embryonic day 7, whereas SphK2 expression is only detectable at embryonic day 11 and increases thereafter. Human SphK1 and SphK2 genes are on chromosomes 17q25.2 and 19q13.2, respectively.

S1P lyases

The S1P lyase (Spl) gene *BST1* (for bestowed of sphingosine tolerance) was first cloned from *S. cerevisiae*: mouse[82] and human[83] homologues were subsequently identified by sequence homology. The gene encoding human Spl is on chromosome 10q21 and is expressed most abundantly in the liver. Spl has one TM near its amino terminus and a conserved pyridoxal-dependent decarboxylase domain that contains several essential cysteine residues[83].

S1P phosphatases

Two genes that encode specific sphingoid base phosphate phosphatases (SPPs) have been identified in *S. cerevisiae* — *LBP1/YSR2/LCB3* and *LBP2/YSR3* (REFS 13,14,84). There are two mammalian SPPs, SPP1 (REF. 16) and SPP2 (REF. 18), which contain 8–10 predicted TMs and are highly specific for sphingoid base 1-phosphates. All SPPs contain three conserved motifs that are similar, yet distinct, to those in the lipid phosphate phosphohydrolase superfamily: motif 1, $KDX_{(4)}PRP$; motif 2, $EYX_{(2)}PSXH$; and motif 3, $LVX_{(3)}RXYXGMHX_{(2)}LD$. Although both mammalian SPPs have similar enzymatic properties and localize to the endoplasmic reticulum, SPP1 expression is highest in placenta and kidney, whereas SPP2 is most abundant in brain, heart, colon, kidney, small intestine and lung. The genes that encode human SPP1 and SPP2 are on chromosomes 14q23.1 and 2q36.3, respectively.

S1P and cell fate decisions

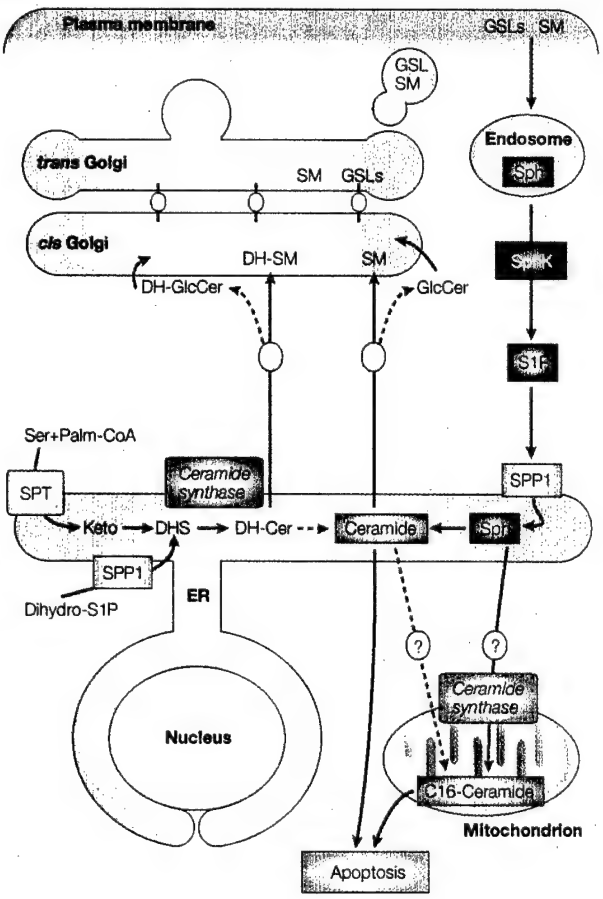
It was originally proposed that apoptotic ceramide was generated by the activation of one or more SPHINGOMYELINASES. Recent studies have also implicated ceramide that is generated from *de novo* sphingolipid biosynthesis[32–34] — predominantly *N*-palmitoylsphingosine (C16-ceramide) — in mitochondrial damage that leads to downstream activation of CASPASES and apoptosis[33]. There is convincing evidence that ceramide synthase and another ceramide biosynthetic enzyme, serine palmitoyltransferase, are activated during apoptosis[33–35] (BOX 2). However, because the ER contains other enzymes that are involved in ceramide biosynthesis as well as SPP1 and SPP2, and SPPs convert S1P to sphingosine (which can be further metabolized to ceramide), this indicates that SPPs could also have an important role in the regulation of ceramide biosynthesis. Indeed, expression of SPP1 altered the dynamic balance between S1P and sphingosine/ceramide in mammalian cells and, consequently, markedly enhanced apoptosis[16,21]. Likewise, the balance between the levels of ceramide and S1P, which is regulated by Lbp1, the yeast ER sphingoid base phosphate phosphatase, is crucial for survival and resistance to environmental stress in yeast[13–15]. This supports the idea that the sphingolipid rheostat is an evolutionarily conserved stress regulatory mechanism.

Ceramide versus dihydroceramide. Interestingly, whereas S1P could induce ceramide biosynthesis (particularly C16-ceramide) and apoptosis in *SPP1* transfectants, dihydro-S1P (which is also a substrate for SPP1 and SPP2) had no effect on ceramide levels, nor did it induce apoptosis[21]. In this regard, several inferences can

Box 2 | **The topology of sphingolipid metabolism and implications for apoptosis**

The *de novo* synthesis of all sphingolipids begins at the cytoplasmic face of the endoplasmic reticulum (ER) — which is where the enzymes that are required for ceramide biosynthesis are located — by the condensation of L-serine (Ser) with palmitoyl CoA (Palm-CoA), a reaction that is catalysed by serine palmitoyltransferase (SPT)[85,86]. In two rapid reactions, the product, 3-ketosphinganine (Keto), is reduced to sphinganine (dihydrosphingosine; DHS) and subsequently acylated by ceramide synthase to form dihydroceramide (DH-Cer). Dihydroceramide is then converted to ceramide by a desaturase[87]. Dihydroceramide and/or ceramide is translocated from the ER to the Golgi apparatus and then converted to sphingomyelin (SM) and dihydrosphingomyelin (DH-SM) by sphingomyelin synthase on the lumenal side of the Golgi, or to glucosylceramide (GlcCer) and dihydroglucosylceramide (DH-GlcCer) on the cytosolic surface of the Golgi[88]. After translocation into the Golgi lumen, glucosylceramide is further converted to lactosylceramide and more complex glycosphingolipids (GSLs). Sphingosine (Sph) produced from degradation of plasma membrane GSLs and SM in the endocytic (Endosome) recycling pathway might be used for formation of ceramide by sequential phosphorylation by sphingosine-1-phosphate (S1P) kinase (SphK) and dephosphorylation by S1P phosphatases (SPPs), or converted into pro-apoptotic C16-ceramide by ceramide synthase in the mitochondria.

The insertion of the *trans* 4,5 double bond into ceramide by the desaturase is an important step because ceramide, and not dihydroceramide, induces apoptosis[32]. Remarkably, although dihydro-S1P is also a substrate for SPP1 — it forms dihydrosphingosine, which is then converted to dihydroceramide by ceramide synthase — it does not cause significant ceramide accumulation or increase apoptosis in *SPP1* transfectants, in contrast to S1P[21]. This indicates that dihydroceramide might be more efficiently used for sphingomyelin and/or glycosphingolipid biosynthesis than ceramide is. Alternatively, the translocation of dihydroceramide to the Golgi might be more rapid than that of ceramide. So, the biosynthetic trafficking of ceramide and dihydroceramide might be different, and vesicular and non-vesicular transport pathways of ceramide versus dihydroceramide might carry out special functions. The figure is modified with permission from REF. 21 © the Rockefeller University Press (2002).



be made: first, SPP1 functions in an unprecedented manner to regulate the biosynthesis of ceramide, which has an essential role in apoptosis; second, the presence or absence of the *trans* double bond in the sphingoid bases dictates their function in the biosynthesis of ceramide; and third, ceramide and dihydroceramide could have different biosynthetic trafficking pathways (BOX 2) or, alternatively, vesicular and non-vesicular pathways of ceramide versus dihydroceramide transport might carry out special functions. As sites of contact have often been observed between the membranes of the ER and mitochondria[36], it is also possible that these could be responsible for delivery of apoptotic ceramide, but not dihydroceramide, to the mitochondria. As dihydroceramides are much less potent than ceramides in the induction of apoptosis, divergence in the regulation of ceramide and dihydroceramide synthesis has important implications, not only for sphingolipid metabolism, but also for their distinct roles in apoptosis. Sphingosine, but not sphinganine (also known as dihydrosphingosine, which has exactly the same structure as sphingosine but lacks the 4,5-*trans* double bond), formed at the ER by SPP1, might be transported to the mitochondria and function as a substrate for ceramide synthase, or for a new mitochondrial ceramidase with reciprocal (ceramide synthase) activity that shows a strong preference for sphingosine over dihydrosphingosine as substrate[37]. This ceramide could induce apoptosis that arises from mitochondrial damage, generation of reactive oxygen species, release of cytochrome *c* and subsequent activation of caspase activity.

S1P regulates ceramide biosynthesis and apoptosis. Although dihydrosphingosine is an intermediate in the biosynthesis of ceramide, it is not converted directly to sphingosine. Sphingosine arises predominantly by the turnover of membrane sphingolipids through the endocytic recycling pathway. Most of the sphingomyelins and glycosphingolipids that are present in slowly dividing cells are in fact made from this recycled sphingosine[38]. In yeast, deletion or mutation of the sphingosine kinase genes, *LCB4* and *LCB5*, or the ER sphingoid base phosphate phosphatase gene, *LBP1*, completely blocked the incorporation of added dihydrosphingosine into sphingolipids[13,14,39]. So, both SphK and SPP are required in this cycle of phosphorylation, uptake into the ER and dephosphorylation for the synthesis of sphingolipids through the endocytic pathway. Likewise, we suggest

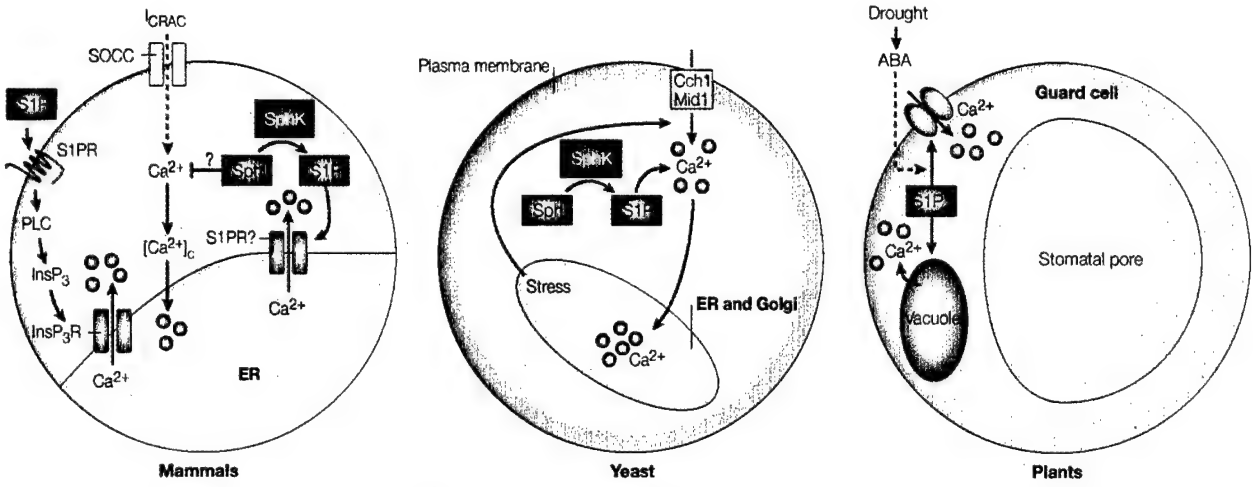


Figure 3 | **S1P regulates calcium homeostasis in plants, yeast and mammals. a** | In mammalian cells, it has been proposed that sphingosine kinase (SphK) converts sphingosine (Sph) — which inhibits the store-operated calcium release-activated calcium current (SOCC) I_{CRAC} — to sphingosine-1-phosphate (S1P), which mobilizes calcium independently of inositol trisphosphate ($InsP_3$) and its receptor ($InsP_3R$). However, the targets (S1P receptors; S1PRs) of S1P on the endoplasmic reticulum (ER) have not yet been identified, as denoted by the question mark. In addition, some S1P receptors on the plasma membrane are coupled to the activation of phospholipase C (PLC), formation of $InsP_3$ and calcium mobilization ($\uparrow[Ca^{2+}]_c$) (FIG. 4). **b** | In yeast, calcium influx in response to stress in the ER is mediated by calcium channels that are composed of Cch1, a homologue of the catalytic subunit of voltage-gated calcium channels, and a regulatory subunit, Mid1. The formation of S1P, which is catalysed by the SphKs Lcb4 or Lcb5, increases calcium influx to replenish secretory organelles with calcium. **c** | In plants, S1P is involved in the signal-transduction pathway that links the drought hormone abscisic acid (ABA) to the release of calcium from intracellular stores, notably the vacuole. It is still unclear how ABA regulates S1P levels and how S1P affects calcium.

that in mammalian cells, sphingosine is formed in late endosomes and lysosomes, then phosphorylated on their cytosolic surfaces by SphK (BOX 2), and the resulting S1P, owing to its more hydrophilic nature, is readily transported to the ER where it is dephosphorylated by SPP1 and/or SPP2 to sphingosine. This sphingosine is subsequently converted to ceramide, predominantly to the C16-ceramide species[16,21], by ceramide synthase. As the overexpression of SPP1, which dephosphorylates S1P, increases *de novo* synthesis of ceramide, it is possible that this increase could result from relief of the negative regulation of serine palmitoyltransferase or ceramide synthase by S1P. One piece of evidence in support of such a hypothesis is that the suppression of sphingoid base synthesis and downregulation of serine palmitoyltransferase by free sphingoid bases requires the conversion of these free bases to sphingoid base-1-phosphates[40]. It is intriguing to speculate that negative regulation by S1P of one of the key enzymes in the *de novo* ceramide biosynthesis pathway might be the mechanism for the pro-survival activity of S1P and its ability to suppress ceramide-mediated apoptosis, and that it might also explain why the relative levels of these interconvertible sphingolipid metabolites are tightly regulated.

PHOSPHOLIPASE C
A phosphoric diester hydrolase that splits the bond between the phosphorus atom and the oxygen atom at C1 of the glycerol moiety of a glycerophospholipid.

S1P and calcium homeostasis

From mammals... More than a decade ago, it was suggested that a sphingosine derivative formed in the ER[41], which was later identified as S1P[42,43], could mobilize calcium from internal sources in an inositol trisphosphate ($InsP_3$)-independent manner. Over the ensuing years, many external stimuli — beginning with crosslinking of the immunoglobulin receptor[44] — that were known to regulate calcium homeostasis without activation of PHOSPHOLIPASE C (PLC), have been shown to stimulate SphK[45,46]. Specific SphK inhibitors block the increases in intracellular calcium concentration that are elicited by these agents, which indicates the importance of SphK activity and, by inference, the generation of S1P (FIG. 3). In addition, the store-operated calcium release-activated calcium current (I_{CRAC}), which is important for replenishing calcium stores, is blocked by sphingosine. So, conversion to S1P, which is catalysed by SphK, would lower sphingosine levels leading to the disinhibition of I_{CRAC}, which would thereby also increase calcium influx[47]. Although at present there are several reports that support the idea that SphK is involved in calcium mobilization, this has become the subject of controversy, because intracellular targets in the ER that are responsible for the effects of S1P have still not been identified. Furthermore, the presence of S1P receptors that are coupled to PLC and calcium mobilization complicates this issue (discussed below). However, it should be remembered that the first studies on the mobilization of calcium in ER preparations by sphingosine showed that it required conversion of sphingosine to S1P, and that S1P itself mobilized calcium in these microsomal membranes in the absence of plasma membrane S1P receptors[43,48,49]. Moreover, S1P has recently been shown to be involved in regulating calcium levels in yeast and higher plants, which do not

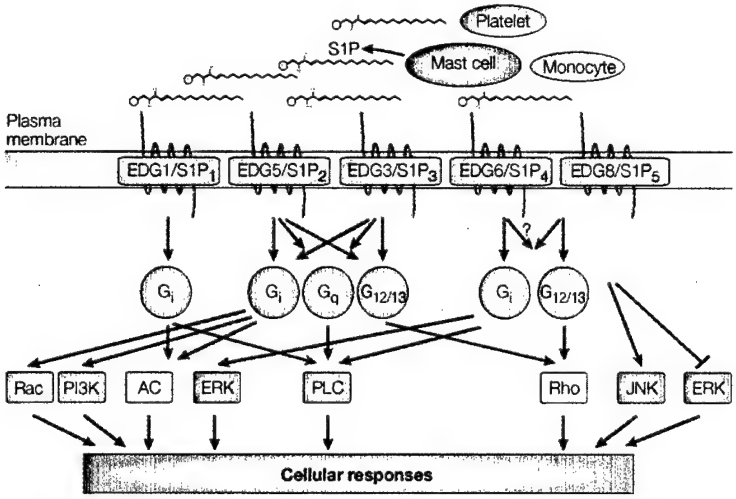


Figure 4 | **S1P is a ligand for five G-protein-coupled receptors.** Sphingosine-1-phosphate (S1P) in serum, which is secreted by mast cells, platelets and monocytes, binds to specific members of the S1P receptor family, which are coupled to different G proteins (for example, EDG1/$S1P_1$ and EDG6/$S1P_4$ couple mainly to G_i; both EDG5/$S1P_2$ and EDG3/$S1P_3$ activate G_i, G_q and $G_{12/13}$; and EDG8/$S1P_5$ is linked to G_i and $G_{12/13}$) leading to activation or inhibition of the indicated downstream signalling pathways. Only a few examples of these pathways are illustrated — in particular, extracellular signal-regulated kinase, ERK; Jun amino terminal kinase, JNK; the small GTPases of the Rho family (Rho and Rac); phospholipase C, PLC; adenylyl cyclase–cyclic AMP, AC; and phosphatidylinositol 3-kinase, PI3K. The reader is referred to recent reviews on signalling downstream of S1P receptors and the G proteins that they are coupled to[22,66].

have S1P receptors, and this indicates that S1P might have a universal intracellular signalling role in calcium regulation (FIG. 3a).

...to yeast and plants. Recent evidence indicates that yeast might use I_{CRAC}-like mechanisms to help replenish secretory organelles with calcium[50]. Calcium influx in response to stress in the ER (which can be induced by perturbations of protein, carbohydrate or lipid biosynthesis in the ER) is mediated by calcium channels that are similar to voltage-gated calcium channels. This calcium influx requires SphKs (Lcb4 and Lcb5), and is inhibited by Spl (Dpl1) and the SPP Lcb3 (REF. 50; FIG. 3b).

In higher plants, STOMATA form pores on leaf surfaces for the exchange of water and gases between the plant and the atmosphere. The plant hormone abscisic acid (ABA), the levels of which increase in response to drought, regulates stomatal pore size by mediating turgor changes in the GUARD CELLS that surround the pore. An increase in the cytosolic concentration of free Ca^{2+} is a common intermediate in many of the pathways that lead to either opening or closure of the stomatal pore. Recently, it was shown that S1P, but not dihydro-S1P, stimulates stomatal closure and Ca^{2+} mobilization in *Commelina communis* guard cells. In addition, the promotion of stomatal closure by ABA was attenuated by a SphK inhibitor, and finally, drought also increased S1P levels[51,52]. These results indicate that S1P is also important in calcium regulation and the control of guard-cell aperture in plants (FIG. 3c). So, it is intriguing to note that S1P regulates calcium both in yeast and plants, particularly in response to harsh environmental conditions.

STOMATA
The pores in the epidermis of plants, in particular in the leaves, through which gaseous exchange occurs.

GUARD CELLS
Cells that are found on the underside of plant leaves, which pair up to form stomata, or leaf pores. Guard cells control the size of the stomata, and so, in turn, regulate gas exchange in the leaf.

G-PROTEIN-COUPLED RECEPTOR
A seven-helix transmembrane-spanning cell-surface receptor that signals through heterotrimeric GTP-binding and -hydrolysing G-proteins to stimulate or inhibit the activity of a downstream enzyme.

HETEROTRIMERIC G PROTEIN
A component of receptor-mediated activation or inhibition of adenylyl cyclase and other second messenger systems.

S1P as an agonist of S1P receptors

Since the first report that S1P was the ligand for the orphan G-PROTEIN-COUPLED RECEPTOR (GPCR) endothelial differentiation gene 1 (EDG1)[53], it has become clear that the most important biological role of S1P is to function as the natural ligand for what has grown into the EDG family of GPCRs, which are now also known as S1P receptors. So far, five members — EDG1/$S1P_1$, EDG5/$S1P_2$, EDG3/$S1P_3$, EDG6/$S1P_4$, and EDG8/$S1P_5$ (REF. 54) — which bind only S1P and dihydro-S1P with high affinity, have been identified. They are ubiquitously expressed and couple to various G proteins that regulate numerous downstream signals (FIG. 4). This endows S1P with the ability to regulate diverse physiological processes, including angiogenesis and vascular maturation[55-58], heart development[59] and immunity[60-62] in a highly specific manner, depending on the relative expression of S1P receptors and G proteins. S1P receptors also differentially regulate the small GTPases of the Rho family, particularly Rho and Rac[56], which are downstream of the HETEROTRIMERIC G PROTEINS and are important for cytoskeletal rearrangements[63] and directed cell movement[64,65]. For example, $S1P_1$ regulates Rac-coupled cortical actin formation[56], and $S1P_2$ and $S1P_3$ activate Rho[65]. $S1P_2$ also blocks Rac activation[65], thereby inhibiting cell movement. These aspects have recently been covered in several comprehensive reviews[22,66,67] and are not discussed at length here. Rather, we focus our attention on a few examples that illustrate the roles of S1P receptors in development, as well as highlighting some recent advances.

S1P in cell migration: heart development. Coordinated cell migration is central to many physiological processes, including embryonic development, organogenesis, wound healing and the immune response. Recent studies indicate that S1P receptors have key roles in these processes. During development, cardiac precursor cells migrate from two patches of tissue to the dorsal midline and merge to form the heart. A mutation in the gene encoding the zebrafish homologue of $S1P_2$ causes 'miles apart' split-heart development (FIG. 5a). This was the first evidence that S1P has a role in cell migration during embryogenesis. Interestingly, the zebrafish Miles-apart protein does not need to be expressed on the migrating heart precursor cells themselves; rather, it is also expressed in PARAXIAL CELLS, which are located on either side of the midline. So, $S1P_2$ signalling might somehow enable these cells to direct cardiac precursor cells to the midline. Surprisingly, however, disruption of *$S1P_2$* in mice did not result in a similar phenotype[68].

S1P in cell migration: vascular development. An essential role for $S1P_1$ in vascular development and maturation was shown by gene disruption in mice. *$S1P_1$*-knockout mice died *in utero* between embryonic day 12.5 (E12.5) and E14.5 owing to vascular abnormalities that were caused by the defective migration of mural cells (vascular smooth muscle cells (VSMCs) and PERICYTES) around nascent blood vessels (FIG. 5b). Fibroblasts obtained from $S1P_1$-null embryos failed to

PARAXIAL CELLS
Cells of a region of the mesoderm, which is known as the paraxial mesoderm, that is adjacent to the notochord. The paraxial mesoderm becomes segmented rostrocaudally to give rise to the somites early in development.

PERICYTE
A support cell of the capillaries. Pericytes are known as smooth muscle cells in larger vessels.

IMMUNOMODULATOR
Any agent that alters the extent of the immune response to an antigen.

LYMPHOPENIA
A decrease in the number of lymphocytes in the blood, which might occur in various diseases.

show Rac activation and were unable to migrate towards S1P[57], indicating that S1P might function directly on $S1P_1$ that is expressed by VSMCs/pericytes to facilitate their recruitment and migration around the vessels. However, the indirect influence of the Miles-apart protein on migrating cells, together with the high level of expression of $S1P_1$ in endothelial cells, raises the possibility that S1P could also stimulate $S1P_1$ that is expressed on endothelial cells, and this in turn would recruit VSMCs[57].

What are the mechanisms by which cells expressing S1P receptors could regulate migration of another cell type? The activation of S1P receptors could upregulate adhesion molecules or cause other changes to the cell surfaces that would then be permissive for the recruitment of migrating cells. Or perhaps S1PR signalling could induce the release of components of the extracellular matrix or of a diffusible signal to attract migrating cells. Although much remains to be learned before this question can be answered definitively, there is evidence in the literature that supports either or both of these possible mechanisms. S1P has been shown to induce Rho-dependent activation of integrins[69], to affect vascular endothelial growth factor (VEGF) signalling[70] and to activate matrix-degrading proteinases[71].

S1P in lymphocyte migration. An unanticipated but important function for S1P receptors in lymphocyte migration and immune responses emerged from studies with the IMMUNOMODULATOR and sphingosine analogue FTY720, which is a drug that shows great potential for human kidney transplantation and the management of chronic autoimmune diseases such as multiple sclerosis. FTY720 elicits LYMPHOPENIA, which results from a reversible redistribution of lymphocytes from the circulation to secondary lymphoid tissues. Two recent reports show that FTY720 is phosphorylated by SphK1 and that the phosphorylated compound is a potent agonist of all S1P receptors except $S1P_2$ (REFS 60,61). Phosphorylated FTY720, which presumably functions through S1P signalling pathways, modulates chemotactic responses and lymphocyte trafficking to divert lymphocytes from inflammatory lesions and graft sites (FIG. 5c).

S1P receptors and receptor crosstalk

The importance of S1P in cell migration, angiogenesis[56], vascular maturation[57], vascular permeability[58] and tumour biology prompted a flurry of studies on its relationship with VEGF, one of the most important growth factors that is involved in the processes of

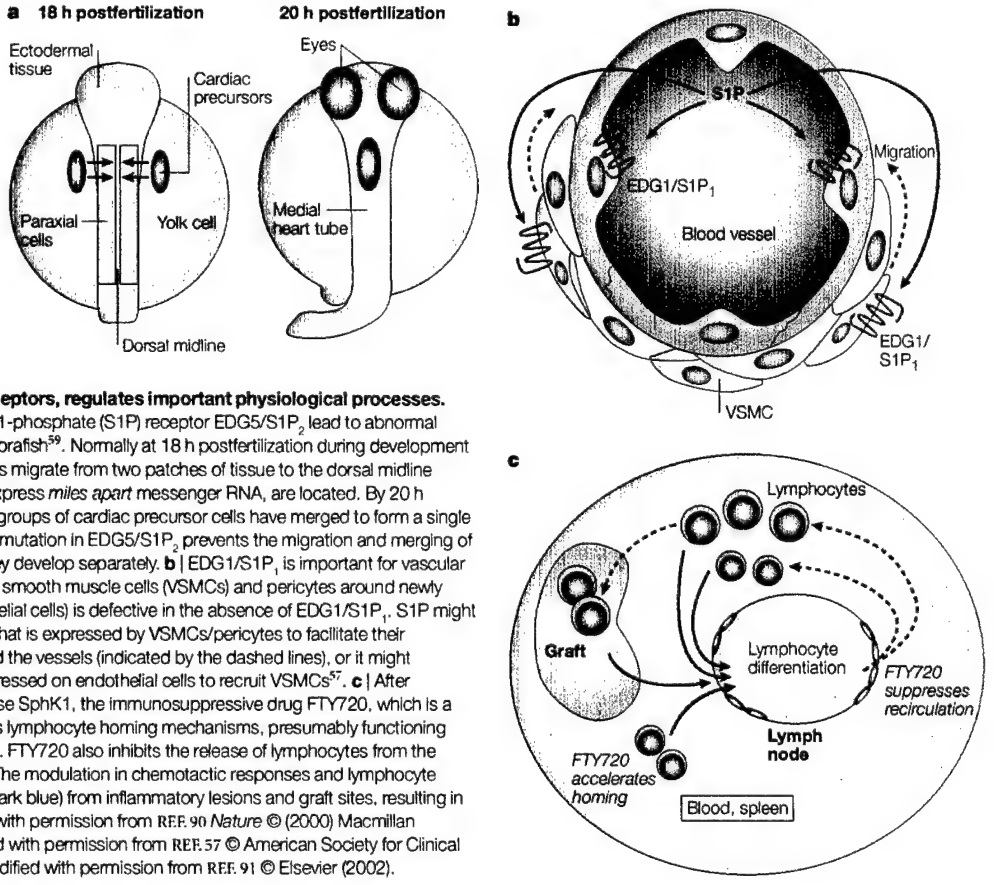


Figure 5 | **S1P, through S1P receptors, regulates important physiological processes.** **a** | Mutations in the sphingosine-1-phosphate (S1P) receptor EDG5/$S1P_2$ lead to abnormal split-heart development in the zebrafish[59]. Normally at 18 h postfertilization during development (left panel), cardiac precursor cells migrate from two patches of tissue to the dorsal midline where the paraxial cells, which express *miles apart* messenger RNA, are located. By 20 h postfertilization, the two bilateral groups of cardiac precursor cells have merged to form a single medial heart tube (right panel). A mutation in EDG5/$S1P_2$ prevents the migration and merging of these two groups of cells and they develop separately. **b** | EDG1/$S1P_1$ is important for vascular maturation. Migration of vascular smooth muscle cells (VSMCs) and pericytes around newly formed endothelium (EC, endothelial cells) is defective in the absence of EDG1/$S1P_1$. S1P might function directly on EDG1/$S1P_1$ that is expressed by VSMCs/pericytes to facilitate their recruitment and migration around the vessels (indicated by the dashed lines), or it might stimulate EDG1/$S1P_1$ that is expressed on endothelial cells to recruit VSMCs[57]. **c** | After phosphorylation by the S1P kinase SphK1, the immunosuppressive drug FTY720, which is a sphingosine analogue, stimulates lymphocyte homing mechanisms, presumably functioning through S1P signalling pathways. FTY720 also inhibits the release of lymphocytes from the lymph node into the circulation. The modulation in chemotactic responses and lymphocyte trafficking diverts lymphocytes (dark blue) from inflammatory lesions and graft sites, resulting in lymphopenia. Part **a** is modified with permission from REF. 90 *Nature* © (2000) Macmillan Magazines Ltd. Part **b** is modified with permission from REF. 57 © American Society for Clinical Investigation (2000). Part **c** is modified with permission from REF. 91 © Elsevier (2002).

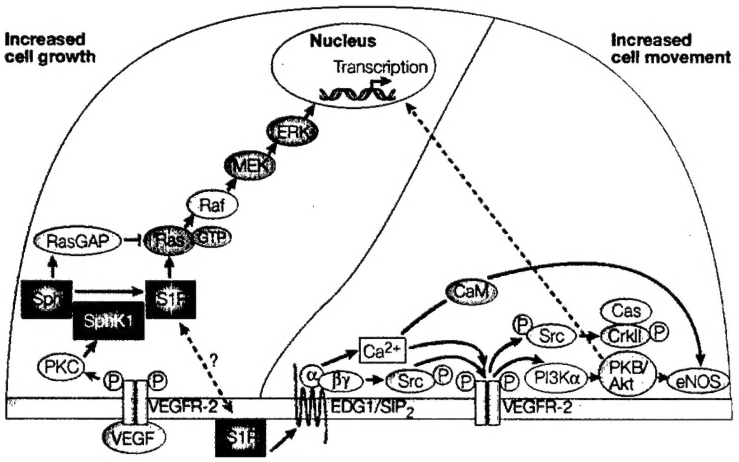


Figure 6 | **Crosstalk of VEGF and S1P signalling.** The crosstalk of sphingosine-1-phosphate (S1P) and vascular endothelial growth factor (VEGF) in cell movement is shown in the right-hand side of the figure (light-blue shading). S1P can activate VEGF receptor-2 (VEGFR-2) in the absence of added VEGF by receptor crosstalk. Ligation of the S1P receptor EDG1/$S1P_1$ and activation of G_i and $G_{\beta\gamma}$, in turn, lead to the activation of components such as Src that result in the phosphorylation of VEGFR-2. This transactivation of VEGFR-2 can then lead to activation of two signalling cascades that are important for movement and vascular remodelling: first, activation of Src-family tyrosine kinase(s) and the adaptor protein CrkII; and second, activation of phosphatidylinositol 3-kinase α (PI3Kα), protein kinase B (PKB)/Akt and endothelial nitric oxide synthase (eNOS), and the formation of nitric oxide (NO). The dashed arrow indicates that PKB/Akt is essential not only for cell migration but also for cell proliferation. In response to G_i-protein-mediated activation of phospholipase C, intracellular calcium (Ca^{2+}) levels increase. Ca^{2+} then complexes with calmodulin (CaM), which also activates eNOS. For simplicity, other known signalling pathways downstream of EDG1/$S1P_1$ (as indicated in FIG. 4) are not shown. The crosstalk of VEGF and S1P in cell growth is shown on the left-hand side of the figure (light yellow shading). After binding VEGF, VEGFR-2 activates protein kinase C (PKC), which then stimulates the S1P kinase SphK1. This increases S1P levels with concomitant decreases in sphingosine (Sph) and inhibition of the GTPase-activating protein, RasGAP[28]. Activation of Ras, in turn, will lead to activation of Raf, MAPK and ERK kinase (MEK), extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and, eventually, to DNA synthesis. The question mark and dashed arrow in this part of the figure indicate that it is still not known whether S1P that is generated intracellularly by VEGF can stimulate S1P receptors to regulate cell movement.

vasculogenesis and angiogenesis. These studies have shown that there is very complex crosstalk between these two molecules. S1P, similar to various agonists of GPCRs, can activate growth factor tyrosine kinase receptors in the absence of added growth factors, which indicates that receptor tyrosine kinases might be activated through receptor crosstalk (which is also known as transactivation).

Implications for angiogenesis. Two recent studies have shown that S1P can transactivate VEGF receptors in endothelial cells (FIG. 6). In the first study, activation of one of the VEGF receptors — VEGF receptor 2 (VEGFR-2/Flk-1) — and the subsequent activation of Src-family tyrosine kinases were required for S1P-induced phosphorylation of the adaptor protein CrkII in human umbilical vein endothelial cells (FIG. 6). In agreement with the established role of CrkII, S1P-promoted membrane ruffling and cell motility were also decreased by an inhibitor of VEGFR-2 (REF. 72). Similarly, S1P transactivated VEGFR-2 on endothelial cells through a signalling pathway that involved G_i, calcium and Src-family tyrosine kinases[70]. Unexpectedly, the transactivation of VEGFR-2 by S1P was independent of the release of endogenous VEGF, because a neutralizing antibody did not block the effect of S1P, in contrast to Src inhibitors. Taken together with other studies, it seems that activation of $S1P_1$ leads to G_i-dependent activation of the β isoform of phosphatidylinositol 3-kinase (PI3Kβ) and PLC. This results in an increased concentration of intracellular calcium, which complexes with calmodulin to activate endothelial nitric oxide synthase (eNOS)[73]. eNOS produces nitric oxide (NO), which has a crucial role in the regulation of vascular tone, vascular remodelling and VEGF-induced angiogenesis. Simultaneously, transactivation of VEGFR-2 is followed by the activation of Src-family tyrosine kinases and CrkII, which are important for membrane ruffling and cell motility. In addition, transactivation of VEGFR-2 results in the activation of protein kinase B (PKB)/Akt (which is regulated by PI3Kα), which also phosphorylates and activates eNOS[70] (FIG. 6). What makes this interplay even more complex is the finding that S1P-stimulated PKB/Akt can bind $S1P_1$ and phosphorylate its third intracellular loop at threonine 236, which is required for Rac activation and chemotaxis that is induced by S1P[74].

Implications for cell growth. Recent studies indicate that VEGF can also stimulate SphK1, and this has ramifications for its mitogenicity[28]. It has long been known that VEGF stimulates endothelial cell growth through protein kinase C (PKC), which leads to the activation of ERK1/2. However, the exact mechanism by which this happens remained elusive until a recent study showed that SphK1 was the missing link between PKC and ERK1/2 (REF. 28). Pharmacological inhibitors, DOMINANT-NEGATIVE SphK1, or SMALL INTERFERING RNA (siRNA) targeted to SphK1 all blocked VEGF-induced, but not epidermal growth factor (EGF)-induced, ERK1/2 activation and DNA synthesis. Incubation of cells with siRNA that specifically targeted SphK1, but not SphK2, blocked the VEGF-induced accumulation of active, GTP-bound Ras. In this study, it was proposed that membrane-associated sphingosine in cells attenuates basal Ras activity by stimulating the activity of Ras GTPase-activating proteins (RasGAPs). VEGF stimulation of PKC and the consequent activation of SphK1 results in the conversion of sphingosine to S1P, which then displaces sphingosine from GAPs (FIG. 6). Overall, this would decrease GAP activity and increase the level of activated Ras–GTP, without influencing the opposing activity of Ras guanine nucleotide exchange factors (RasGEFs)[28], leading to activation of the ERK/mitogen-activated protein kinase (MAPK) pathway and cell division (FIG. 6).

An important question is whether the S1P that is produced by VEGF-mediated stimulation of cellular SphK1 activates S1P receptors to activate Ras and ERK/MAPK, or whether it functions as an intracellular second messenger. Several lines of evidence have been proposed in support of the latter possibility[28].

DOMINANT-NEGATIVE
A defective protein that retains interaction abilities and so distorts or competes with normal proteins.

SMALL INTERFERING RNA (siRNA). Short (21–23mers) sequences of double-stranded RNA that are used in RNA interference, a process by which the expression of homologous genes is silenced through degradation of their cognate mRNA.

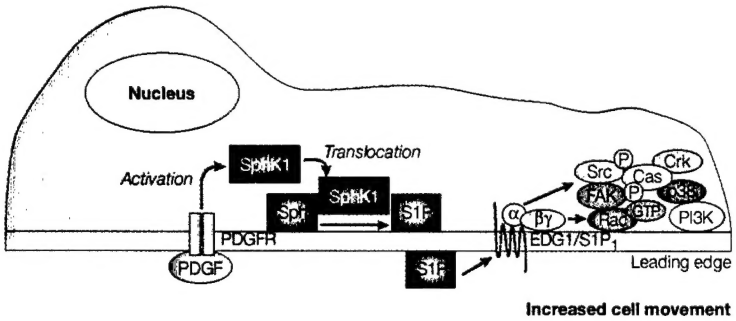


Figure 7 | **Transactivation of EDG1/S1P$_1$ and PDGF-directed cell movement.** The sphingosine-1-phosphate (S1P) kinase SphK1 is diffusely distributed in the cytosol of unstimulated cells, but platelet-derived growth factor (PDGF) rapidly induces its activation and translocation to membrane ruffles, where it phosphorylates membrane-associated sphingosine (Sph) to form S1P. S1P binds to the receptor EDG1/S1P$_1$, leading to activation of several downstream signals, such as focal adhesion kinase (FAK), Src, p38, Rac, phosphatidylinositol 3-kinase (PI3K), Cas and Crk, that are important for cell locomotion. As explained in the text, this crosstalk has ramifications for the migration of pericytes and smooth muscle cells around newly formed blood vessels.

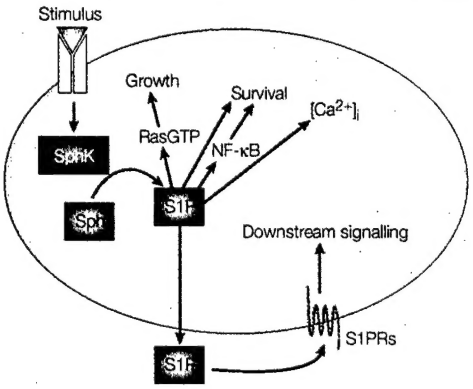
First, PERTUSSIS TOXIN blocks S1P-induced, but not VEGF-induced, ERK1/2 activation. Second, S1P–S1P-receptor activation of ERK is blocked by dominant-negative Ras, whereas VEGF activation of ERK/MAPK is insensitive. Finally, no secretion of S1P could be detected. So, it seems that signalling of VEGF and of extracellular S1P are distinct, which indicates that the intracellular S1P generated by VEGF leads to activation of downstream signalling without engaging S1P receptors[28].

Implications for movement. Reciprocal mechanisms of transactivation have been reported to be important in cell movement that is directed by platelet-derived growth factor (PDGF)[61]. Ligation of the PDGF tyrosine kinase receptor by PDGF activates and translocates SphK1 to the plasma membrane. This gives rise to the spatially restricted formation of S1P, and, in turn, activates S1P$_1$, which is essential for PDGF-directed cell movement (FIG. 7). This indicates that intracellularly generated S1P might signal 'inside–out' through its cell-surface receptors (BOX 3). S1P receptors are linked to the G-protein-mediated activation of downstream signals, such as Rac and p38, to regulate cytoskeletal rearrangements and focal adhesion turnover that is modulated by the tyrosine kinases focal adhesion kinase (FAK) and Src, which are important for cell locomotion[75] (FIG. 7). However, it is still not clear whether such inside–out signalling contributes to the effects of S1P on cell growth and suppression of apoptosis (BOX 3).

How do external stimuli stimulate SphK1?

Several recent studies have investigated the mechanisms regulating the activation of SphK1 by various stimuli, where this occurs in the cell, and whether it leads to the release of S1P extracellularly. As SphK1 is predominantly a cytosolic enzyme, whereas its substrate sphingosine is generated in membranes, it is not surprising that translocation to membranes is a common feature of SphK1 activation. For example, SphK1 is translocated to the leading edge of cells during PDGF-induced cell

Box 3 | **S1P signalling inside and out**



Diverse external stimuli, particularly growth and survival factors and chemoattractants — including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNF-α), nerve growth factor, epidermal growth factor, basic fibroblast growth factor, IgE/antigen, phorbol ester, vitamin D$_3$, ATP, fMLP, oxidized low density lipoprotein, bradykinin, endothelin-1, cyclic AMP, acetylcholine, lysophosphatidic acid, prosaposin and even sphingosine-1-phosphate (S1P) itself through S1P receptors (S1PRs; for a review, see REF. 22) — stimulate the S1P kinase SphK1 to generate intracellular S1P. S1P might have dual functions. First, it can function in an autocrine or paracrine fashion to stimulate S1P receptors that are present on the cell surface of the same or nearby cells. Coupling of S1P receptors to diverse G proteins leads to activation of numerous downstream signalling pathways (FIG. 4). This type of crosstalk has been shown for the PDGF receptor, which transactivates the S1P receptor EDG1/S1P$_1$, leading to the modulation of components of downstream pathways, including Src, focal adhesion kinase, and Rac, which are important for PDGF-directed cell migration[76]. It is not known whether other components of signalling pathways, such as phospholipase C (which regulates calcium levels), or phosphatidylinositol 3-kinase (which activates protein kinase B/Akt), might also be regulated in this manner. It is not even clear how S1P is secreted and presented to S1P receptors. Second, S1P might also have intracellular functions to regulate calcium levels, survival and growth. For example, crosslinking of the antigen receptor FcεRI stimulates SphK1 and S1P production, which then mobilizes calcium from internal stores independently of inositol trisphosphate formation[44,46]. TNF-α and other cytokines stimulate SphK1 leading to the activation of the transcription factor nuclear factor κB (NF-κB), which is essential for the prevention of apoptosis[30]. The potent angiogenic factor VEGF stimulates SphK1 to produce S1P, which mediates VEGF-induced activation of Ras and consequently, extracellular-signal regulated kinase/mitogen-activated protein kinase signalling and cell growth[28].

PERTUSSIS TOXIN
A mixture of proteins that is produced by *Bordetella pertussis*. It causes the persistent activation of G_i proteins by catalysing the ADP-ribosylation of the α-subunit.

PHORBOL ESTERS
Polycyclic esters that are isolated from croton oil. The most common is phorbol myristoyl acetate (PMA, also known as 12,13-tetradecanoyl phorbol acetate or TPA). They are potent co-carcinogens or tumour promoters because they mimic diacylglycerol, thereby irreversibly activating protein kinase C.

PHOX HOMOLOGY DOMAIN
A domain that is similar in function to pleckstrin homology domains. It has an affinity for certain phosphorylated phospholipids.

migration[76]. In addition, activation of PKC by the PHORBOL ESTER phorbol 12-myristate 13-acetate or VEGF phosphorylates and activates SphK1. However, whereas translocation of SphK1 to the plasma membrane was accompanied by increased secretion of S1P, which allows for autocrine/paracrine signalling in response to phorbol ester[77], no secretion of S1P was induced by VEGF[28]. In another type of activation, an elegant study identified a tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2)-binding motif of SphK1 that mediated the interaction between TRAF2 and SphK. This resulted in the activation of SphK1, which in turn was required for TRAF2-mediated activation of NF-κB and the anti-apoptotic effect of TNF[30]. Furthermore, two other unrelated SphK1-interacting proteins have recently been identified by yeast two hybrid screens. One has some similarity to a family of protein kinase A anchor proteins and affects the activity of SphK1 (REF. 78). The other, RPK118 (for ribosomal S6 kinase-like protein with two pseudo kinase domains), which also contains a PHOX HOMOLOGY DOMAIN and specifically binds to phosphatidylinositol 3-phosphate, induced the translocation of SphK1 to early endosomes[79]. It is tempting to speculate that this translocation might allow it to phosphorylate sphingosine that is produced in the salvage pathway (BOX 2).

New clues from plants

A new study shows that SphK is involved in both ABA-mediated inhibition of stomatal opening and the promotion of stomatal closure in *Arabidopsis thaliana*, by regulating guard-cell inward K^+ channels and anion channels[52]. Surprisingly, S1P regulates stomatal apertures and guard-cell ion-channel activities in wild-type plants, but not in the absence of the sole prototypical heterotrimeric G protein α-subunit (GPA1), which indicates that heterotrimeric G proteins might be downstream targets for S1P. Of particular interest, the putative GPCR of *A. thaliana*, GCR1, has no sequence homology to any of the conserved S1P receptors. Therefore, the S1P signal in guard cells might be transduced by the direct interaction of S1P with GPA1 or by unidentified proteins that stimulate heterotrimeric G proteins independently of GPCRs. As the intracellular targets of S1P in mammalian cells have not yet been identified, these studies in plants might provide clues to the enigmatic intracellular action of S1P in mammalian cell growth.

Conclusion and perspectives

Research on the sphingolipid metabolite S1P has expanded tremendously in the past few years. In this review, we have highlighted how S1P levels are regulated and how S1P is able to regulate so many physiological processes. Appropriately, considering its name, it is still a riddle why intracellularly generated S1P can signal 'inside–out' to regulate cell movement, but in the few examples studied so far, S1P receptors seem not to have a role in its growth and survival actions. There is no doubt that deciphering the complex interplay between S1P signalling inside and out and determining how it is transported into and out of cells will uncover many more hidden secrets. Studies in lower organisms, such as yeast, slime mould, plants, worms and flies, should provide clues to the ancient roles of S1P. Future work will unravel the mystery of the many hues of this simple lipid S1P.

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